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APPLICATION NUMBER: *60/059,353*
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PRIORITY DOCUMENT

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A/PRV

Practitioner's Docket No. 22722/OMP.PRV **PATENT**

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of: Rikihisa, et al.

For: OUTER MEMBRANE PROTEIN OF EHRLICHIA CANIS AND EHRLICHIA CHAFFEENIS

Box Provisional Patent Application
Assistant Commissioner for Patents
Washington, D.C. 20231

COVER SHEET FOR FILING PROVISIONAL APPLICATION
(37 C.F.R. § 1.51(2)(i))

WARNING: "A provisional application must also include a cover sheet identifying the application as a provisional application. Otherwise, the application will be treated as an application filed under § 1.53(b)(1)." 37 C.F.R. § 1.53(b)(2)(i).

NOTE: "A complete provisional application does not require claims since no examination on the merits will be given to a provisional application. However, provisional applications may be filed with one or more claims as part of the application. Nevertheless, no additional claim fee or multiple dependent claims fee will be required in a provisional application." Notice of December 5, 1994, 59 FR 63951, at 63953.

"Any claim filed with a provisional application will, of course, be considered part of the original provisional application disclosure." Notice of April 14, 1995, 60 Fed. Reg. 20,195, at 20,209.

NOTE: "A provisional application shall not be entitled to the right of priority under § 1.55 or 35 U.S.C. 119 or 365(a) or to the benefit of an earlier filing date under § 1.78 or 35 U.S.C. 120, 121 or 365(c) of any other application." 37 C.F.R. § 1.53(b)(2)(ii).

NOTE: "No information disclosure statement may be filed in a provisional application." 37 C.F.R. § 1.51(2)(b). "Any information disclosure statements filed in a provisional application would either be returned or disposed of at the convenience of the Office." Notice of December 5, 1994, 59 FR 63591, at 63594.

NOTE: "No amendment other than to make the provisional application comply with all applicable regulations, may be made to the provisional application after the filing date of the provisional application." 37 C.F.R. § 1.53(b)(2).

CERTIFICATION UNDER 37 C.F.R. 1.10*
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I hereby certify that this correspondence and the documents referred to as attached therein are being deposited with the United States Postal Service on September 19, 1997 (date), in an envelope as "EXPRESS MAIL POST OFFICE TO ADDRESSEE" service under 37 C.F.R. 1.10, Mailing Label Number EM238368245US addressed to the: Assistant Commissioner for Patents, Washington, D.C. 20231.

Pamela A. Docherty
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WARNING: A provisional application may be abandoned by operation of 35 U.S.C. 111(b)(5) on a Saturday, Sunday, or Federal holiday within the District of Columbia, in which case, a nonprovisional application claiming benefit of the provisional application under 35 U.S.C. 119(e) must be filed no later than the preceding day that is not a Saturday, Sunday, or Federal holiday within the District of Columbia. Notice of April 14, 1995, 60 Fed. Reg. 20,195 at 20,202.

This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 C.F.R. 1.53(b)(2).

1. The following comprises the information required by 37 C.F.R. § 1.51(a)(2)(i)(A):

2. The name(s) of the inventor(s) is/are (37 C.F.R. § 1.51(a)(2)(i)(B)):

NOTE: While the name or names of the inventors are required in order to accord a provisional application a filing date, a provisional application is not required to be signed by the inventor or the assignee. No oath or declaration is required. Presumably, most provisional applications will be filed by a registered practitioner without a power of attorney being filed. Notice of December 5, 1994, 59 FR 63591, at 63594.

NOTE: "The naming of inventors for obtaining a filing date for a provisional application is the same as for other applications. A provisional application filed with the inventors identified as 'Jones et al.' will not be accorded a filing date earlier than the date upon which the name of each inventor is supplied unless a petition with the fee set forth in § 1.17(i) is filed which sets forth the reasons the delay in supplying the names should be excused. Administrative oversight is an acceptable reason. It should be noted that for a 35 U.S.C. 111(a) application to be entitled to claim the benefit of the filing date of a provisional application the 35 U.S.C. 111(a)[,] application must have at least one inventor in common with the provisional application." Notice of April 14, 1995, 60 Fed. Reg. 20,195, at 20,209.

The term "invention" is typically used to refer to subject matter which applicant is claiming in his/her application. Because claims are not required in a provisional application, it would not be appropriate to reference joint inventors as those who have made a contribution to the "invention" disclosed in the provisional application. If the "invention" has not been determined in the provisional application because no claims have been presented, then the name(s) of those person(s) who have made a contribution to the subject matter disclosed in the provisional application should be submitted. Section 1.45(c) states that "if multiple inventors are named in a provisional application, each named inventor must have made a contribution, individually or jointly, to the subject matter disclosed in the provisional application." All that § 1.45(c) requires is that if someone is named as an inventor, that person must have made a contribution to the subject matter disclosed in the provisional application. When applicant has determined what the invention is by the filing of the 35 U.S.C. 111(a) application, that is the time when the correct inventors must be named. The 35 U.S.C. 111a) application must have an inventor in common with the provisional application in order for the 35 U.S.C. 111(a) application to be entitled to claim the benefit of the provisional application under 35 U.S.C. 119(e). Notice of April 14, 1995, 60 Fed. Reg. 20,195, at 20,208.

"If all the names of the actual inventor or inventors are not supplied when the specification and any required drawings are filed, the provisional application will not be given a filing date earlier than the date upon which the names are supplied unless a petition, with the fee set forth in § 1.17(q), is filed, which sets forth that the reasons for the delay in supplying the names should be excused." 37 C.F.R. § 1.53(b)(2).

	GIVEN NAME	MIDDLE INITIAL OR NAME	FAMILY (OR LAST) NAME
1.	Yasuko		Rikihisa
2.	Noris		Ohashi
3.			

3. Address(es) of the inventor(s), as numbered above (37 C.F.R. § 1.51(a)(2)(i)(C)):

1. 1120 Woodman Drive, Worthington, Ohio 43210
2. 1210 Chambers Road, Columbus, Ohio 43212
3. _____

4. The title of the invention is (37 C.F.R. § 1.51(a)(2)(i)(D)):

Outer Membrane Protein of Ehrlichia Canis and Ehrlichia Chaffeenis

5. The name, registration, and telephone number of the practitioner *(if applicable)* is (37 C.F.R. § 1.51(a)(2)(i)(E)):

Name of practitioner: Pamela A. Docherty

Reg. No. 40,591 Tel. (216) 622-8416

(complete the following, if applicable)

☐ A power of attorney accompanies this cover sheet.

6. The docket number used to identify this application is (37 C.F.R. § 1.51(a)(2)(i)(F)):

Docket No.: 22727/OMP.PRV

7. The correspondence address for this application is (37 C.F.R. § 1.51(a)(2)(i)(G)):

CALFEE, HALTER & GRISWOLD LLP, 1400 McDonald Investment Center, 800 Superior Avenue, Cleveland, Ohio 44114

8. Statement as to whether invention was made by an agency of the U.S. Government or under contract with an agency of the U.S. Government.
(37 C.F.R. § 1.51(a)(2)(i)(H))

This invention was made by an agency of the United States Government, or under contract with an agency of the United States Government.

☒ No.

☐ Yes.
The name of the U.S. Government agency and the Government contract number are: _____

9. Identification of documents accompanying this cover sheet:

A. Documents required by 37 C.F.R. §§ 1.51(a)(2)(ii)–(iii):

Specification: No. of pages 71

Drawings: within the specification No. of sheets 27

B. Additional documents:

☐ Claims: No. of claims _____

Note: A complete provisional application does not require claims. 37 C.F.R. § 1.51(a)(2).

☐ Power of attorney

☐ Small entity statement

☐ Assignment

☐ Other

NOTE: Provisional applications may be filed in a language other than English as set forth in existing § 1.52(d). However, an English language translation is necessary for security screening purposes. Therefore, the PTO will require the English language translation and payment of the fee mandated in § 1.52(d) in the provisional application. Failure to timely submit the translation in response to a PTO requirement will result in the abandonment of the provisional application. If a 35 U.S.C. 111(a) application is filed without providing the English language translation in the provisional application, the English language translation will be required to be supplied in every 34 U.S.C. 111(a) application claiming priority of the non-English language provisional application. Notice of April 14, 1995, 60 Fed. Reg. 20,195, at 20,209.

10. Fee

The filing fee for this provisional application, as set in 37 C.F.R. § 1.16(k), is $150.00, for other than a small entity, and $75.00, for a small entity.

☐ Applicant is a small entity.

NOTE: "A verified statement in compliance with existing § 1.27 is required to be filed in each provisional application in which it is desired to pay reduced fees." Notice of April 14, 1995, 60 Fed. Reg. 20,195, at 20,197.

11. Small entity statement

☐ The verified statement(s) that this is a filing by a small entity under 37 C.F.R. §§ 1.9 and 1.27 is(are) attached.

12. Fee payment being made at this time

☒ Not enclosed

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OR

Signature of practitioner

Date: September 19, 1997

Reg. No.: 40,591

Tel.: (216) 622-8416

Customer No.:

Pamela A. Docherty

(type or print name of practitioner)

Calfee, Halter & Griswold LLP
1400 McDonald Investment Center

P.O. Address

800 Superior Aveneu
Cleveland, Ohio 44114

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Pamela A. Docherty
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22727/OMP.PRV

ABSTRACT

Canine ehrlichiosis consists of an acute and a chronic phase. The acute phase is characterized by fever, serous nasal and ocular discharges, anorexia, depression, and loss of weight. The chronic phase is characterized by severe pancytopenia, epistaxis, hematuria, blood in feces in addition to more severe clinical signs of the acute disease. The disease occurs throughout the world and is especially prevalent in Southeast Asia. Canine ehrlichiosis is found throughout the United States. If treated early during the course of the disease, dogs respond well to doxycycline. However, chronically infected dogs do not respond well to the antibiotic. Therefore, early diagnosis is very important for treating canine ehrlichiosis. The indirect fluorescent antibody (IFA) test that uses the etiologic agent, Ehrlichia canis, as antigen, has been the most frequently used for laboratory diagnosis of canine ehrlichiosis. The IFA test has, however, serious limitations: 1) the IFA test is subjective and produces false positive or negative results by an inadequately trained examiner; 2) the IFA test requires a special equipment to perform the test. A simpler, more rapid, and objective serodiagnosis is desirable for canine ehrlichiosis. We report here that for the first time we cloned and expressed 30 kDa, the major antigenic protein of E. canis. Using the recombinant E. canis 30 kDa protein as antigen we developed sensitive and simple dot blot assay which detects E. canis infection in dogs. Since this antigen is the major surface antigen of E. canis we predict that this recombinant protein is also useful for vaccine preparation against canine ehrlichiosis.

INTRODUCTION

Canine ehrlichiosis consists of an acute and a chronic phase. All susceptible dogs exhibit the acute signs, but hemorrhagic signs that characterize the severe chronic form are less predictable and tend to occur in certain breeds, especially German shepherds, 2 to 4 months after the earlier febrile episode. Within 7 to 11 days following inoculation with infective blood, susceptible dogs develop signs of acute disease consisting of fever, serous nasal and ocular discharges, anorexia, depression, and loss of weight. Hematologic findings in the acute phase include thrombocytopenia and nonregenerative anemia (12). Leukocyte counts are variable and sometimes increase during the first 2 to 3 weeks of infection. Leukopenia, if it occurs, probably relates to increased sequestration or destruction of circulating leukocytes. Increases in serum ALT occur, especially in the acute phase of the disease. Dogs that reach the severe pancytopenic stage of chronic infection have a poor prognosis despite therapy. Death is usually caused by complications of secondary infection or hemorrhage.

Canine ehrlichiosis is transmitted by the brown dog tick, Rhipicephalus sanguineus, transstadially but not transovarially, leading to the conclusion that canids, not ticks, are reservoirs. Vertebrate hosts for E. canis appear to be restricted to members of the family Canidae. The jackal, coyote, and fox have been infected with E. canis experimentally. Donatien and Lestoquard reported experimental infection of a monkey, Macacus inuus, but their findings have not been confirmed.

The indirect fluorescence antibody (IFA) test is the clinical laboratory test currently most frequently used for canine ehrlichiosis. My laboratory has developed enzyme-linked

immunosorbent assay (ELISA) and Western immunoblot procedure and a single step and a nested PCR for canine ehrlichiosis. All of these methods are cumbersome and require special equipment to perform. IgM and IgG IFA and ELISA did not vary substantially in detecting early stages of infection. Western blot procedure provides information of antibody reactions to individual E. canis antigenic proteins. By western blot analysis of both experimentally and naturally infected dogs, the 30 kDa antigen of E. canis was identified as the major antigen reacted with all infected dogs. My laboratory cloned and expressed the major antigen of E. canis 30 kDa major outer membrane protein.

The dot-immunoblot assay has been developed for diagnosis of rickettsial organisms. The assay is relatively simple and does not require special equipment as long as membrane strips coated with a high quality antigen are available. Advantage of the recombinant E. canis 3OkDa major antigen over the purified E. canis antigen is its purity and consistency in quality. Therefore, we evaluated our recombinant E. canis 3OkDa antigens by western immunoblot and by dot blot assay.

DATA

Figure 11 demonstrates that we obtained the recombinant clone which expresses a large quantity of E. canis major antigen 30 kDa (Fig. 11A). The size of the recombinant protein is slightly smaller than the native protein due to our technical manipulation for its efficient expression. Fig. 1B compared uninfected dog macrophage DH82 cells (which we used as negative control antigen in Fig. 2), purified whole E. canis, sonication-insoluble recombinant E. canis 30 kDa protein (this means partially purified--this is >90% pure), and affinity chromatography-purified recombinant E. canis 30 kDa protein (100% pure). The amount of 30 kDa protein expressed in recombinant E. coli is much greater than the amount naturally present in E. canis. This proves that we obtained recombinant E. canis 30 kDa antigenic protein and established the method to purify it.

Figure 12 demonstrates that this recombinant E. canis 30 kDa antigenic protein is highly antigenic and has much stronger antigen than native E. canis 30 kDa protein. My LABORATORY has over 2,000 dog sera tested by indirect fluorescent antibody (IFA) test using E. canis as antigen. Representative 7 dog sera with different IFA titer was used for this study. Dog macrophage DH82 cells and E. coli were used as control antigen. The purified E. canis and the recombinant E. canis 30 kDa antigenic protein were compared for their reactivity with dog sera of deferent IFA titers by Western immunoblot analysis, As seen clearly in Fig. 2 IFA negative dog sera has no reaction to any antigens used. Anti-E. canis sera of greater than 1:20 IFA titers, diluted at 1: 1,000 all reacted strongly with native and recombinant 30 kDa antigens. This is the major antigen recognized by the infected dogs. The reactivity of recombinant antigen is much stronger dm that of native E. canis antigen. The Western blot results are summarized in Table 1.

Figure 13 shows that using dog sera of different IFA titers, we compared the effect of preabsorption of dog sera with E. coli, since most dogs have an antibody against E. coli. As seen in Fig. 3 our dot blot assay using semi-purified recombinant 30 kDa antigen can detect all IFA

positive sera including that of 1:20 IFA titer. The negative dog sera was negative. Preabsorption of dog sera with E. coli is not required, since there is no nonspecific reaction of dog sera to the recombinant antigen preparation. There was also no difference in sensitivity and specificity between chromatography purified and semi-purified recombinant antigens in the dot blot assay. This proves that our recombinant E. canis antigen is useful for serodiagnosis of canine ehrlichiosis.

Finally since recombinant 30 kDa antigen is the major surface antigen of E. canis, I predict this is effective as the component of canine ehrlichiosis vaccine.

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Rikihisa, Y. (1996) Ehrlichiae (Plenary Session). In: Proceedings of the 5th International Symposium on Rickettsiae and Rickettsial Diseases (Bratislava, Slovak Republic). Slovak Academy of Sciences, Bratislava, Slovak Republic: International Society of Rickettsiae and Rickettsial Diseases, pp 272-286.

Table 1. Reactivities of purified *E. canis* and the recombinant (r) P30 by western blot analysis with clinical dog sera.

Numbers of clinical dog sera	IFA titers	Antigens			
		E. coli host (I)[a]	DH82 cells	Purified *E. canis*	rP30 of *E.canis* (I)[b]
1	< 1:20	-	-	-	-
2	1:20	-	-	+	+
3	1:40	-	-	+	++
4	1:80	-	-	++	+++
5	1:2,560	-	-	+++	++++
6	1:5,120	-	-	+++	++++
7	1:10,240	-	-	++++	+++++

[a] Sonication-insoluble fraction (1) of *E. coli* host.
[b] Sonication-insoluble fraction (1) of the recombinant clone (partially purified rP30).

ABSTRACT

Five major proteins in 23- to 29-kDa range were identified in an outer membrane fraction prepared from *Ehrlichia chaffeensis,* human ehrlichiosis agent. The NH_2-terminal amino acid sequence of an in immunodominant 28-kDa outer membrane protein (OMP)-1, one of the major OMPs, chemically determined was homologous to that of *Cowdria ruminantium* 32-kDa major antigen protein (MAP)-1. The DNA fragment including the 28-kDa outer membrane protein gene *(omp)-1* amplified by polymerase chain reaction (PCR) was cloned, sequenced, and expressed. The NH_2-terminal amino acid sequence of *E. chaffeensis* 23-kDa OMP was found to be similar to a part of the predicted amino acid sequence of *omp-1.* The sequences of 25- and 27-kDa OMPs at the NH_2 termini were homologous. Rabbit and mouse anti-recombinant-OMP-1 antibodies cross-reacted strongly with 25-kDa, 28-kDa (native OMP-1), and 29-kDa OMPs of *E. chaffeensis.* These findings suggest that *E. chaffeensis* in a tissue culture coexpresses several homologous proteins in the 23- to 29-kDa range that have common antigenic epitopes and homologous amino acid sequences, i.e., that these proteins are members of a multi-sized protein antigen family. In an experiment of *E. chaffeensis-challenge,* immunization of recombinant OMP-1 protected mice from the ehrlichial infection. The result suggests that 28-kDa OMP-1 is a candidate for development of vaccine and diagnostic antigen.

Ehrlichia chaffeensis, which causes human ehrlichiosis, is an obligatory intracellular bacterium of monocytes and macrophages and belongs to the family *Rickettsiaceae.* Human ehrlichosis is a newly recognized and emerging rickettsial disease in the United States. The first case was reported in 1987 as a human infection with *Ehrlichia canis,* the causative agent of canine ehrlichiosis, since the patient serum strongly reacted with *E. canis* antigen by indirect fluorescent antibody test (1). However, a new but similar organism, *E. chaffeensis, was* isolated in 1990 at the Centers for Disease Control (CDC), Atlanta, Ga., from a patient from Fort Chaffee, Ark. (2). Since 1987, over 400 cases of human ehrlichiosis detected primarily by serological means have been reported in 30 states (2-4).

So far, few molecular-level studies of the pathogenesis and intracellular parasitism of *E. chaffeensis* have been carried out. This lack is probably due to the difficulty of obtaining a sufficient quantity of viable *E. chaffeensis* in its pure form, since this organism grows slowly in monocyte cell lines and is very fragile. Moreover, it is impossible to apply to obligate intracellular bacteria such as ehrlichiae the gene manipulation techniques generally used with facultative intracellular or extracellular bacteria. Hence, our initial effort was concentrated on identifying the ehrlichial major outer membrane proteins, cloning and expressing their genes, and analyzing the functions of these proteins by using the gene products.

Recently, several proteins, including the heat shock protein (HSP) 60 homolog of *E. chaffeensis,* were identified by Western immunoblot analysis as major antigens in infected human and experimentally inoculated dogs (5-8). Among these antigens, - 30-kDa protein antigens are predominant in both *E. chaffeensis and E. canis* and are antigenically cross-reactive between these two *Ehrlichia* spp. (6). However, the nature and localization of these protein antigens and their roles in host immunity are unknown. We reported here the identification of five 23- to 29-kDa major proteins in the outer membrane fraction of E. *chaffeensis,* and the cloning, sequencing, and expression of a 28-kDa protein gene, one of major outer membrane proteins (OMP). Immunization of mice with recombinant 28-kDa protein protected mice from *E.*

chaffeensis infection. Surprisingly, these five major proteins were found to belong to a multi-sized protein antigen family whose members have antigenic epitopes and amino acid sequences in common.

MATERIALS AND METHODS

Organisms and Purification. *E. chaffeensis and* <u>*E. canis*</u> cultivated in DH82 dog macrophage cell line (6), were purified by Percoll density gradient centrifugation as described elsewhere (9) but with some modifications. Heavily infected cells (approximately 6 x 10^8) were harvested and centrifuged at 800 x g. The pellet was suspended in SPK buffer (0.2 M sucrose and 0.05 M potassium phosphate buffer, pH 7.4) and homogenized on ice with 30 strokes by using a Dounce homogenizer. DNase 1, RNase A (Sigma, St. Louis, Mo.), and $MgCl_2$ (final concentrations of 50 µg/ml, 10 µg/ml, and 2 mM, respectively) were then added to the homogenate and incubated for 10 min on ice. The homogenate was centrifuged at 400 x g three times for 10 min per time. The nuclease reaction in the supernatant was terminated by adding EDTA (final concentration, 5 mM). The organisms in the supernatant were mixed with Percoll (Sigma) (final Percoll concentration, 32 to 35 %) and centrifuged at 61,900 x g for 30 min. The lower layer was harvested, mixed with SPK buffer, and centrifuged at 11,670 x g for 10 min. The pellet was resuspended in SPK buffer and centrifuged twice to remove Percoll. The protein content of purified ehrlichiae was determined by the Coomassie blue dye-binding assay (Bio-Rad Laboratories. Richmond, CA.) as described (10).

Preparation of Ehrlichial Outer Membrane. The procedure for *Orientia tsutsugamushi* (11) was followed with modifications. Purified organisms (100 µg of each) were suspended with 10 MM sodium phosphate buffer containing 0.1% Sarkosyl (Sigma), 50 µg each of DNase I and RNase A, and 5 mM $MgCl_2$. After incubation at 37°C for 30 min and termination of reaction of these nucleases by the addition of EDTA (final concentration, 15 mM), proteins in the soluble supernatant and the insoluble precipitate, separated by centrifugation at 10,000 x g for I h, were analyzed by SDS-PAGE as described elsewhere (12). Prestained protein molecular size markers were purchased from GIBCO-BRL (Grand Island, NY) and Bio-Rad.

Electron Microscopy. Transmission electron microscopy was performed by a procedure described elsewhere (13). Ultrathin sections (60 nm) were stained with uranyl acetate and lead citrate and observed with a Philips 300 electron microscope at 60 kV.

NH_2-Terminal Amino Acid Sequence Analysis. Proteins of the Sarkosyl-insoluble precipitate from 400 µg of whole organisms were separated by a reversed-discontinuous SDSPAGE (2.5-cm-long 17% gel on top of 11-cm-long 12% gel) and were electrophoretically transferred (at 300 mA for 2 h using a Bio-Rad transblot cell) to a ProBlot™ membrane (Applied Biosystems, Foster City, CA) in 10 mM 3-[cyclohexylaminol-1-propanesulfonic acid (CAPS) (Sigma) and 10% methanol (pH 11). After the proteins on the membrane were stained with 0.1% amido black for 1 min, the bands were cut and individually analyzed with an Applied Biosystems protein sequencer (model 470). A homology search was carried out with data bases of the

GenBank, Swiss Plot, PDB and PIR by using the software basic local alignment search tool (14) in the BLAST network service (the National Center for Biotechnology Information, Bethesda, MD).

Cloning and Sequencing of PCR-Amplified 28-kDa outer membrane protein gene *(omp)-1.* Genomic DNA of *E. chaffeensis* was isolated from purified ehrlichiae as described elsewhere (15). Synthetic oligonucleotides containing 5'-terminal sequences for *EcoRI, BamHI, and NotI* restriction endonucleases were prepared by Bioserve (Laurel, MD). The 5' oligonucleotide (f28pl primer) included the DNA sequence coding for the NH_2-terminal region of *E. chaffeensis* 28-kDa outer membrane protein (OMP)- 1 (amino acid positions 6 to 12), chemically determined, and for the *EcoRI and* BamHI restriction endonuclease sites [5'-CG GGA TCC GAA TTC GG(ATGC) AT(ATC) AA(TC)GG(ATGC)AA(TC)TT(TC)TA -3,]. The 3' oligonucleotide (r28pl primer) included the DNA sequence coding for the COOH-terminal region conserved between *Cowdria ruminantium* MAP-1 and *Anaplasma marginate* major surface protein (MSP)-4 with the addition of a *NotI* restriction endonuclease site [5'- AGC GGC CGC TTA (AG)AA (TC)A(CG) (AG)AA (CT)CT T(CG)C TCC -3'1. PCR amplification was carried out with a Perkin-Elmer Cetus DNA Thermal Cycler (model 480) by the standard procedure. The 0.8-kb amplified product of almost-full-length *omp-1* was cloned in the pCR™II vector of a TA cloning kit as described by the manufacturer (Invitrogen Co., San Diego, CA). Both DNA strands of a DNA insert of five recombinant clones were sequenced by a dideoxy termination method with an Applied Biosystems 373A DNA sequencer. One clone was designated pCRIIOMP-1. DNA sequences were analyzed with the DNASIS program (Hitachi Software Engineering Co., Ltd., Yokohama, Japan).

Overexpression of OMP-1. The DNA fragment of *omp-1* purified from EcoRI- and NotI-digested pCRIIOMP-1 after agarose gel electrophoresis was ligated into dephosphorylated, EcoRI- and NotI-digested pET29a (Novagen, Inc., Madison, WI). *Escherichia coli* NovaBlue (Novagen, Inc.) was transformed with recombinant pET29a. A plasmid preparation of pET29a with *omp-1* from transformed NovaBlue was then used to transform *E. coli* BL21(DE3)pLysS, which has a very low transforming capacity. The induction of the recombinant protein was performed by a procedure described elsewhere (16). A clone, omp-l-pET29a-BL21(DE3)pLysS host, was designated as pET29aOMP-1.

Sera and Werstern Immunoblot Analysis. Convalescent-phase serum from a patient with clinical signs of human ehrlichiosis was provided by the CDC, Atlanta, Ga. (6). For preparation of rabbit anti-recombinant OMP-1 serum, the area of the expressed protein band after SDS-PAGE of pET29aOMP-1 cells was excised without Coomassie blue staining. The gel was minced in phosphate-buffered saline, pH 7.4, and mixed with an equal volume of Freund's incomplete adjuvant (Sigma), and the mixture was subcutaneously injected into a rabbit four times at 2-week intervals (I mg of protein each time). Anti-E. *chaffeensis* antibody titer was determined by indirect immunofluorescence assay (IFA) as described elsewhere (17). The IFA titers of the patient serum and the rabbit anti-recombinant OMP-1 serum against *E. chaffeensis* antigen were 1:2,560 and 1:1,280, respectively.

Western immunoblotting was performed by a procedure described elsewhere (18). Alkaline phosphatase-conjugated affinity-purified anti-human, -rabbit or -mouse

immunoglobulin G (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD) were used at a 1:1,000 or 1:2,000 dilution as a secondary antibodies.

Immunization of Mice and *E. Chaffeensis*-Challenge. A band of recombinant OMP-1 separated from pET29aOMP-1 by SDS-PAGE was excised, minced, and mixed with an equal volume of Freund's incomplete or complete adjuvant. Nine BALB/c male mice (6 weeks old) were divided into two groups. Five mice were intraperitoneally immunized totally four times at 10-day intervals; twice with a mixture of the minced gel with recombinant OMP-1 (30 to 40 μg of protein per mouse each time) and incomplete adjuvant, and twice with a mixture of the recombinant protein (the same amount as before) and complete adjuvant. As noninimunized mice, another four mice were intraperitoneally injected with a mixture of the minced gel without protein and the respective adjuvants. For *E. chaffeensis*-challenge, approximately 10^7 infected DH82 cells were disrupted by sonication in serum-free DMEM (GIBCO-BRL) and centrifuged at 200 x g for 5 min. The supernatant was adjusted to a final volume of 5 ml, and 0.3 ml was inoculated intraperitoneally into each mouse at 10 days after the last immunization. After 5 days, the mice were sacrificed and the blood specimens were collected for detection of *E. chaffeensis* DNA by PCR.

The infectivity of *E. chaffeensis* in the inoculum was assessed by the infected cell counting units (ICU) method (19). Serial 10-fold dilutions (0.15 ml each) of the inoculum were added to uninfected DH82 cells in a Lab-Tek tissue culture chamber (Nunc Inc. Naperville, IL). After 3 days of incubation, the percentage of infected cells was determined. Total cell number per well was determined by counting trypsinized (0.1%)-uninfected DH82 cells. The ICU in 0.3 ml of the original suspension was determined by the following formula: ICU= (percentage of infected cells) x (1/100) x (total cell number) x (1/dilution) x (0.3/0.15).

Detection of *E.* chaffeensis 16S RDNA in Ehrlichia-Challenged Mice. Approximately 1 ml of blood collected in an EDTA tube from each mouse was centrifuged at 600 x g for 15 min. The buffy coat and blood plasma obtained were used for DNA preparation and western blot analysis, respectively. Total DNA was prepared from 0.2 ml of the buffy coat with a QIAamp blood kit (QIAGEN, Inc., Chatsworth, CA). This DNA was used as the template in the PCR for detection of *E. chaffeensis* DNA. The primers used were HEl (5'-CAATTGCTTAT-AACCTTTTGGT TATAAAT-3') and HE3 (5'- TATAGGTACCGTCATTATCTTCCCTAT - 3'), which are specific for *E. chaffeensis* 16 RDNA and which generated the expected 389-bp fragment (20). The PCR was performed by the procedure as described elsewhere (21).

RESULTS

Identification of Major Outer Membrane Proteins of *E.* chaffeensis and Analysis of the NH2-Terminal Amino Acid Sequences. In a preliminary test for isolation of an ehrlichial outer membrane, purified *E. chaffeensis* Arkansas was treated with different concentrations (0.05, 0.1, and 0.2%) of Sarkosyl. SDS-PAGE analysis of the resultant soluble supernatant and insoluble precipitate showed that with 0.1% Sarkosyl treatment (Fig. 1A), the insoluble precipitate contained several 23- to 29-kDa major proteins (considered as a 30-kDa cluster), whereas the 55-kDa protein (HSP60 homolog [9]) and several other minor proteins were in the

soluble supernatant. Treatment of *E. chaffeensis with* 0.05% Sarkosyl resulted in a PAGE profile similar to that obtained with 0.1% Sarkosyl treatment and 0.2% Sarkosyl solubilized almost all proteins (data not shown). With 0.1% Sarkosyl treatment of *E. canis*, the result was similar to that obtained with *E. chaffeensis* (Fig. 1B). Transmission electron microscopy revealed ehrlichial outer membrane vesicles of various sizes in the 0.1% Sarkosyl-insoluble precipitate of *E. chaffeensis* (Fig. 2). These results indicate that treatment of ehrlichiae with Sarkosyl at a concentration of 0.1% (or 0.05 %) effectively separates the ehrlichial outer membrane proteins from other proteins. To analyze in detail the major proteins in the 30-kDa cluster, proteins in the 0.1% Sarkosyl-insoluble precipitate were separated by a reversed-discontinuous SDS-PAGE (Fig. 3). The results obtained showed that this cluster consists of at least five major proteins with molecular masses of 29, 28, 27, 25, and 23 kDa on PAGE. The NH_2-terminal amino acid sequences of all of these five major proteins were chemically determined. The amino acid sequence of OMP-1 is NH2-DPAGSGINGNFYISGKYMP-COOH. The NH_2-terminal amino acid sequence of 23-kDa protein was homologous to the sequence from amino acids 54 to 68 predicted from open reading frame (ORF) of *E. chaffeensis omp-1* described bellow (Fig. 4 and Fig. 5A). The sequences of 25-kDa and 27-kDa proteins at the NH_2 termini were homologous (Fig. 5B). On the other hand, we recently cloned genes encoding Gen proteins homologous to OMP-1, but not identical, from *E. chaffeensis* genomic DNA with a labeled PCR-amplified *omp-1* as a probe. The NH_2-terminal amino acid sequence of 29-kDa protein determined chemically was homologous to a part of the predicted amino acid sequence of the Gen-1, one of Gen proteins (Fig. 5C). This result suggests that *E. chaffeensis* in DH82 cells coexpresses several similar proteins with different molecular masses.

DNA and Predicted Amino Acid Sequence of PCR-Amplified *omp-1*. An almost-full-length fragment of *omp-1* amplified with f28pl and r28pl primers was cloned (pCRIIOMP-1) and sequenced (Fig. 4). The insert fragment of a pCRIIOMP-1 clone encoded a protein consisting of 251 amino acid residues (including both PCR primer regions). The predicted molecular mass of this protein was 27,685 Da. The protein encoded on the insert does not contain five amino acid residues (DPAGS) that correspond to the NH_2 terminus of the native OMP-1. The predicted amino acid sequence, including DPAGS, revealed that this protein is rich in Gly (11.32 mol %) and Ser (10.54 mol %), and that its estimated isoelectric point is 5.06.

Overexpression of OMP-1 and Its Immunoreactivity. A clone, pET29aOMP-1, that was constructed with an insert of pCRIIOMP-1 overexpressed a large amount of recombinant OMP-1 (Fig. 7A). The pET29aOMP-1 clone produced a protein larger than native OMP-1 because it included 35 peptides from the pET29a vector upstream of Gly at the NH2 terminus of OMP-1 derived from the pCRIIOMP-1 insert. The predicted molecular mass of the expressed protein was 31,063 Da. The recombinant OMP-1 was recognized strongly by convalescent-phase serum from a patient with clinical signs of human ehrlichiosis (Fig. 7B, lane 2). The patient serum cross-reacted predominantly to 28-kDa native OMP-1 and 29-kDa protein of purified *E. chaffeensis* (lane 3) and to the 30-kDa major protein of *E. canis* (lane 1). Using-rabbit anti-recombinant OMP-1 serum, Western immunoblot analysis revealed three strongly cross-reactive proteins of 29 kDa, 28 kDa (native OMP-1), and 25 kDa in E. *chaffeensis*, and 30-kDa protein in *E. canis* (Fig. 9). These are not degradation products of larger proteins, since the NH2-terminal amino acid sequences of these proteins were not identical to any parts of the

OMP-1 amino acid sequences. These facts suggest that E. *chaffeensis* in DH82 cells coexpresses several homologous proteins with different molecular masses, i.e., that these proteins are members of a multi-sized protein antigen family.

Protection against *E. chaffeensis* Challenge in Mice Immunized with Recombinant OMP-1 and Protein Antigens Recognized by the Immunized Mice. The anti-E. *chaffeensis* IFA titer of sera collected from the retroorbital plexus of immunized mice was 1:160 at the time of challenge. Sera from the control group of mice were negative. Recombinant OMP-1-immunized mice and noninimunized mice were challenged with an *E. chaffeensis* inoculum of 1.28 x 106 ICU per mouse. Protection was assessed by PCR, which detected a 16S rDNA fragment specific to *E. chaffeensis* (389 bp) in the buffy coat from each mouse at 5 days postchallenge. Day 5 is the earliest time at which establishment of ehrlichial infection can be investigated by PCR without the influence of residual DNA from the ehrlichiae used as the challenge. An *E. chaffeensis*-specific fragment was observed in all nonimmunized mice but not in any immunized mice, showing that immunization with recombinant OMP-1 protects mice from ehrlichial infection (Fig. 9).

By Western blot analysis *chaffeensis-challenged* mice which were previously sera from two immunized mice predominantly reacted with 29- and 28-kDa (native OMP-1) proteins in purified *E. chaffeensis* (Fig. 10A and B) while sera from the other two immunized mice were reacted with three major proteins of 29 kDa, 28 kDa (native OMP-1), and 25 kDa (Fig. IOC and E). The antigen profile in panel C was similar to that of the rabbit anti-recombinant OMP-1 serum in Fig 8. The reactivity of the native OMP-1 on blots was strongest among all reactive proteins. The blood plasma from one immunized mouse (Fig. IOD) was reactive to recombinant OMP-1 but not to any proteins of *E. chaffeensis*, although the plasma was IFA positive with *E. chaffeensis* antigen and the mouse was protected against the ehrlichial infection. It is unknown why even native OMP-1 did not react with the blood plasma of this mouse. However, these results showed clearly that the antibody response is different in each mouse despite of utilization of an inbred strain for this study. The blood plasma from all four noninimunized mice did not react to any proteins in 30-kDa cluster in the Western blot analysis (data not shown), indicating that immunoreaction to 25, 28 and/or 29 kDa proteins in immunized mice is induced by the immunization with recombinant OMP-1 rather than by the challenge with live *E. chaffeensis*. Thus, the presence of immunocross-reactivities of 25-, 28-, and 29-kDa proteins with blood plasma from immunized mice supports our conclusion that these proteins are members of multi-sized protein antigen family of *E. chaffeensis*.

DISCUSSION

In this study, we identified five 23- to 29-kDa *E. chaffeensis* major proteins in outer membrane enriched by 0. 1 % Sarkosyl treatment and characterized a PCR-amplified omp-I gene encoding one of the major proteins, 28-kDa OMP-1. The relationships among the five major outer membrane proteins, 23 kDa, 25 kDa, 27 kDa, 28 kDa (OMP-1), and 29 kDa are summarized as follows. (i) The 23-kDa major protein has the similar amino acid sequence to that of 28-kDa OMP-1. (ii) The 25-kDa major protein has a common antigenic epitopes with 28-kDa OMP-1. (iii) The 27-kDa major protein possesses the similar NH2-terminal amino acid sequence to that of 25-kDa protein. (iv) The 29 kDa major protein has a common epitopes with 28-kDa

OMP-1. Accordingly, we defined these proteins as a multi-sized protein antigen family, and the 28-kDa OMP-1 is a member of the family. In fact, we have succeeded in cloning several DNA fragments with ORFs similar but not identical, to that of *omp-1* from *E. chaffeensis* genomic DNA by using labeled-PCR-amplified *omp-1* as a probe. The cloned 3.6-kb fragment contained tandemly repeated 5'-truncated, two complete, and 3'-truncated ORFs, all of which are homologous to that of *omp-1;* that is, *omp-1* has multiple gene copies. Gen-1 is one of copies, which possesses a homologous sequence to NH_2-terminal amino acid sequence of 29-kDa protein. The presence of these multigene copies strongly supports our conclusion that *E. chaffeensis* coexpresses multiple homologous proteins.

We looked for in other *Ehrlichia* spp. proteins reactive with rabbit anti-E. *chaffeensis* recombinant OMP-1 serum. Rabbit anti-recombinant OMP-1 serum cross-reacted with three proteins of the homologous strain of *E. chaffeensis* and the 30-kDa protein of *E. canis* in group 1. We recently found that the *E. canis* 30-kDa protein gene may also have multiple gene copies. The predicted amino acid sequences of partial ORFs in the two DNA fragments of *E. canis,* which were cloned after PCR amplification with two different primer pairs corresponding to the sequence *of omp-1,* were similar but not identical to each other and both were homologous to the amino acid sequence of *omp-1.*

The question is why such various homologous outer membrane proteins of approximately 30 kDa exist in *E. chaffeensis and E. canis.* Our previous and preliminary studies led us to speculate as follows. Previously, we observed that acute-phase sera (before 30 days postinoculation) from several *E. canis-infected* dogs reacted strongly with a 30-kDa protein but weakly with a 31 kDa protein. However, with chronic-phase serum (after 60 days postinoculation) from the same infected dog, the 31-kDa protein reacted strongly and the reactivity of 30-kDa protein decreased (31). These results suggest that the ehrlichiae may regulate the expression of homologous proteins on their surfaces in order to elude host immune recognition. On the other hand, our preliminary study found that several ^{125}I- labeled outer membrane proteins of ~30-kDa of *E. chaffeensis* bound to paraformaldehyde-fixed DH82 cells. This suggests that the OMP-1 family may consist of proteins that participate in the attachment of ehrlichiae to eukaryotic host cells. Consequently, the conserved amino acid sequences in these multiproteins may be necessary for attachment of the host cells, and the antigenic polymorphism may be required for eluding host immune recognition.

We demonstrated that recombinant OMP-1 protects mice from *E. chaffeensis* infection, suggesting that this protein may be a candidate for development of a vaccine and a diagnostic antigen for the ehrlichiosis.

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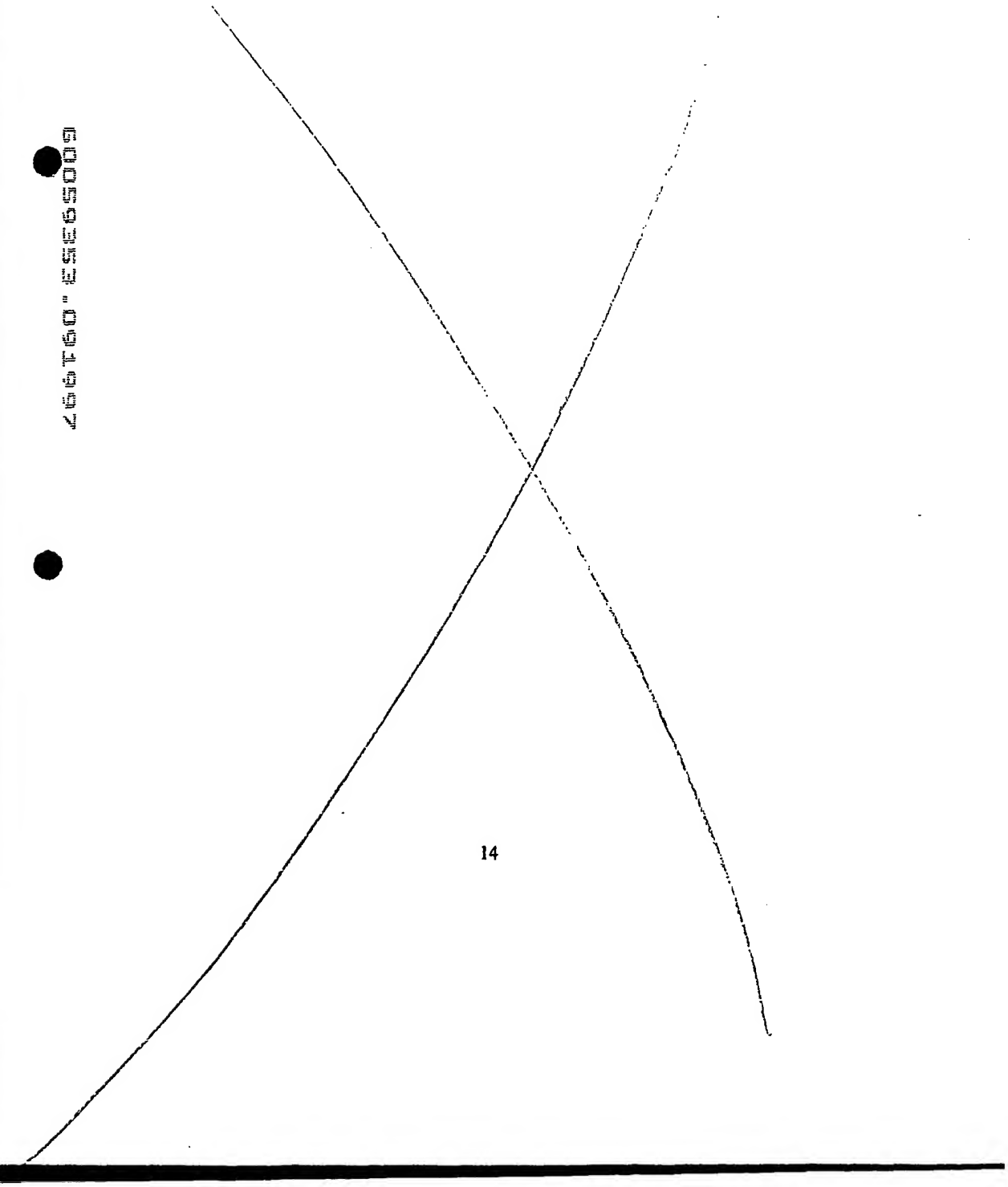


FIGURE LEGENDS

FIG. 1. SDS-PAGE of the insoluble precipitate and the soluble supernatant fraction after 0. 1 % Sarkosyl treatment of purified *E. chaffeensis (A)* and *E. canis (B)*. Proteins at 20 gg of purified whole organism lysates (lane 2), 7 to 13 *ug* of Sarkosyl-insoluble precipitate enriched with the outer membrane (lane 3), and 7 to 13 *ug* of Sarkosyl-soluble supernatant (lane 3) were stained with Coomassie blue after SDS-PAGE. Molecular markers are shown in lane I and indicated in kilodaltons. Brackets indicate a 30-kDa cluster of major outer membrane proteins.

FIG. 2. Electron microscopy of the insoluble precipitate after 0.1% Sarkosyl treatment of the whole *E. chaffeensis* organism. Note outer membrane vesicles of various sizes. (Bar = μm).

FIG. 3. Major outer membrane proteins in the 30-kDa cluster resolved from the Sarkosyl-insoluble precipitate of *E. chaffeensis* by a reversed-discontinuous SDS-PAGE. An SDS-polyacrylamide gel consisting of a 17% gel on top of a 12% gel was used. The Sarkosyl-insoluble precipitate (40 μg per lane) enriched with outer membrane derived from purified *E. chaffeensis* was blotted onto a nitrocellulose sheet and stained with amido black (lanes 1-6). Five protein bands of 23 to 29 kDa on every lane of this sheet were cut, and the NH,-terminal amino acid sequence of each protein was analyzed. Molecular masses of representative major outer membrane proteins are indicated in kilodaltons.

FIG. 4. DNA sequence of pCRIIOMP-1 insert and the predicted amino acid sequence of the ORF. Amino acid residues underlined at amino acid positions 1 to 20 and 53 to 68 are the NH_2-terminal amino acid sequence of native OMP-1 determined chemically and the sequence homologous to the NH_2-terminal amino acid sequence of 23-kDa protein respectively. Five amino acid residues at the NH_2 terminus of native OMP-1, which were not included in the ORF of the pCRIIOMP-1 insert, are indicated by boldface type. Arrows indicate the annealing positions of the primer pair designed for PCR amplification of *omp-1*.

FIG. 5. Characteristics of the NH_2-terminal amino acid sequences of native 23-, 25-, 27-, and 29-kDa major OMPs of *E. chaffeensis*. Similarities were seen in the sequences between 23-kDa protein and OMP-1 (A), in the NH2-terminal sequences between 25-kDa and 27-kDa proteins (B), and in the sequence between 29-kDa protein and Gen-I (C). Identical and conserved amino acids are indicated by colons and periods, respectively, and a gap is indicated by a dash. Amino acids are denoted by the one-letter amino acid code.

FIG. 6. Alignment of deduced amino acid sequences of *E. chaffeensis OMP-1*, *C. ruminantium* MAP-1, and *A. marginate* MSP-4. Aligned positions of amino acids identical to those in *E. chaffeensis* OMP- I are indicated by asterisks. Gaps indicated by dashes are introduced for optimal alignment of all three proteins.

FIG. 7. Overexpression of *E. chaffeensis OMP-1* (A) and its immunoreactivity with convalescent-phase serum from a patient with human ehrlichiosis *(B). (A)* Lysates of pET29a-transformed *E. coli* BL21(DE3)pLysS (20 μg, lane 2) and pET29aOMP-1 transformed *E. coli* BL2 I (DE3)pLysS (20 μg, lane 3) which were harvested at 4.5 h after induction by Isopropyl-ß-D-thiogalactopyranoside were stained with Coomassie blue after SDS-PAGE. An arrowhead indicates overexpressed OMP-1. (B) Proteins from SDS-PAGE gels containing 10 [ig of purified *E. canis* lysate (lane 1), 8 μg of pET29aOMP-1-transformed *E.coli* BL21(DE3)pLysS

(lane 2), and 10 μg of purified *E. chaffeensis* lysate (lane 3) were transferred to a nitrocellulose sheet and incubated with a 1:1,000 dilution convalescent phases serum from a patient. Molecular masses are shown in kilodaltons.

FIG. 8. Western blot analysis with rabbit anti-recombinant *E. chaffeensis OMP-1* serum. Samples subjected to SDS-PAGE were 10 μg of pET29a-transformed *E. coli* (lane 1), 10 jig of pET29aOMP-1 -transformed *E. coli* (lane 2), 20 μg of purified whole *E. chaffeensis* lysate (lane 3), and 20 μg of purified whole *E. canis* lysate (lane 4). These proteins from SDSPAGE gel were transferred to nitrocellulose sheet and incubated with the 1:300 dilution of the rabbit anti-recombinant OMP-1 serum which was preabsorbed twice with pET29a-transformed *E. coli* at 37°C for 1 h per time. Molecular masses are shown in kilodaltons.

FIG. 9. PCR amplification of a 16S RDNA fragment specific for *E. chaffeensis* in the ehrlichia-challenged mice which were previously immunized with a recombinant OMP-1 or nonimmunized. Template DNA's were prepared from blood buffy coats (0.2 ml) of all challenged mice. An arrow shows the *E. chaffeensis*-specific 16S rDNA fragment (389 hp) obtained by PCR amplification. As a template for the positive control, total DNA from DH82 cells infected with *E. chaffeensis* was used (lane 1); for the negative control, a PCR reaction without template was carried out (lane 2). Four nonimmunized (lanes 3-6) and five immunized mice (lanes 7-1 1) that were injected with a mixture of adjuvants and minced polyacrylamide gel without and with recombinant OMP-1, respectively, were challenged with 1.28 x 10^6 ICU of *E. chaffeensis.* A DNA 1-kb ladder is shown in lane 12.

FIG. 10. Western blot analysis with blood plasma obtained from the five *E. chaffeensis*-challenged mice which were previously immunized with recombinant *E. chaffeensis* OMP-1 as shown in Fig. 9. Antigens subjected to SDS-PAGE were 10 μg of pET29aOMP-1 -transformed *E. coli* (lane 1) and 20 μg of purified *E. chaffeensis* lysate (lane 2). These proteins after SDS-PAGE were transferred to a nitrocellulose sheet and incubated with the 1:200 dilution of the blood plasma from each mouse corresponding to lane 7 to I 1 in Fig. 9 (A to E). The blood plasma from four nonimmunized mice in lane 3 to 6 of Fig. 9 was not reactive to any proteins in 30-kDa cluster of *E. chaffeensis* (data not shown). All blood plasma were preabsorbed twice with pET29a-transformed *E. coli* at 37°C for 1 h per time. Molecular masses are shown in kilodaltons.

SUMMARY OF THE INVENTION

In accordance with the present invention, isolated proteins from the outer membrane of Erlichia chafeenis are provided. One of the outer membrane proteins, designated OMP-1 has a molecular weight of about 28 kDa; another outer membrane protein has a molecular weight of about 23 kDa protein; another outer membrane protein has a molecular weight of about 25 kDa protein; another outer membrane protein has a molecular weight of about 27 kDa protein, and another outer membrane protein has a molecular weight of about 29 kDa.. The isolated outer membrane proteins from E. chaffeenis, particularly OMP-1, are immunogenic and are thus, useful for preparing antibodies. Such antibodies are useful for immunolabeling isolates of E. chafeenis and for detecting the presence of E. chafeenis in body fluids, tissues, and particularly in the monocytes and macrophages of individuals, including but not limited to individuals exhibiting symptoms of human ehrlichiosis. The isolated outer membrane proteins, particularly OMP-1A, are also useful for detecting antibodies to E. chafeenis in the blood of individuals with clinical signs of human ehrlichiosis. The isolated outer membrane protein, particularly OMP-1, are also useful in raising antibodies to assist in protecting against ehrlichiosis and in a vaccine for protecting against ehrlichiosis.

In accordance with the present invention an isolated outer membrane protein from Erlichia canis is also provided. This protein, designated has a molecular weight of about 30 kDa. This protein is immunogenic and is, thus, useful for preparing antibodies that are useful for immunolabeling isolates of E. canis. This protein is also useful for diagnosing canine ehrlichiosis and in a vaccine for protecting mammals, particularly members of the family Canidae, most particularly dogs, from canine enhrlichiosis.

The present invention also provides nucleotide sequences encoding proteins, OMP-1 OMP-1A, OMP-1B, OMP-1C, OMP-1D, OMP-1E, and OMP-1F. The present invention also provides nucleotide sequences encoding the 30 kDa outer membrane protein from E. canis. Such sequences are useful for preparing the outer membrane proteins, particularly large amounts of highly purified proteins.

The present invention also relates to a method of using the nucleotide sequences which encode OMP-1A, OMP-1C, OMP-1D, OMP-1E , OMP-1 F, and the 30 kDa outer membrane

protein from E. canis to prepare these proteins or fragments, particularly immunogenic fragments thereof.

The present invention also relates to a method the isolated recombinant proteins made by such methods.

The present invention is also relates to an assay for diagnosing canine ehrlichiosis using the 30 kDa outer membrane protein of the present invention.

Isolated Polynucleotides Encoding OMP-1,OMP-1A, OMP-1B, OMP-1C, OMP-1D, OMP-1F and the OMP from E. Canis

In one aspect, the present invention, provides isolated polynucleotides that encode the outer membrane proteins, OMP-1OMP-1A, OMP-1B, OMP-1C, OMP-1D, OMP-1F from E. chafeenis and the OMP of approximately 30 kDa from E. Canis or immunogenic fragments thereof.

. The polynucleotide may be single stranded or double stranded. The polynucleotide may be a DNA or RNA molecule, preferably a DNA molecule, and comprises a sequence which codes for the respective outer membrane protein. Preferably, the polynucleotide encodes at least the mature form of outer membrane protein. The polynucleotide optionally further comprise a leader sequence and encode an outer membrqane preprotein that is processed and secreted from cells as the mature protein. The polynucleotide of the present invention may also be fused in frame to a marker sequence which allows for purification of the corresponding outer membrane protein.

In one embodiment, the polynucleotide for OMP-1 encodes a protein comprising the amino acid sequence shown in FIG. 4A or 4B (Section A) or an allelic variant thereof. In one embodiment the polynucleotide for OMP-1A, OMP-1B, OMP1-C. OMP1-D, OMP-1D, OMP1-F encodes a protein comprising the amino acid sequence shown in Fig 8 (Section B). In one embodiment, the polynucleotide which encodes the 30 kDa protein from E. canis. Examples of such nucleic acid sequences are shown in Figs 4, 8 and 14. The present invention also relates to polynucleotide comprising a sequence having at least 80%, preferably at least 90%, more preferably at least 95% , most preferably at least 97% identity or complementarity with the nucleotide sequences shown in Fig. 4A, 4B, Fig 8, and Fig. 14. The polynucleotide of the present invention further relates to polynucleotide sequences which hybridize under stringent conditions to the nucleotide sequence shown in Figs 4, Fig 8, and Fig. 14.

The polynucleotides of the present invention are useful for producing the outer membrane proteins of E. chafeenis and E. canis. For example, an RNA molecule encoding the outer membrane protein is used in a cell-free translation systems to prepare the outer membrane protein. Alternatively, a DNA molecule encoding the outer membrane protein is introduced into an expression vector and used to transform cells. Suitable expression vectors include for example chromosomal, nonchromosomal and synthetic DNA sequences, e.g., derivatives of SV40, bacterial plasmids, phage DNAs; yeast plasmids, vectors derived from combinations of plasmids and phage DNAs, viral DNA such as vaccinia, adenovirus, fowl pox virus, and pseudorabies. The DNA sequence is introduced into the expression vector by conventional procedures.

Accordingly, the present invention also relates to recombinant constructs comprising one or more of the sequences as broadly described above. The constructs can be in the form of a vector, such as a plasmid, phagemid, or viral vector, into which a sequence that encodes the outer membrane protein has been inserted.

In the expression vector, the DNA sequence which encodes the outer membrane protein is operatively linked to an expression control sequence, i.e., a promoter, which directs mRNA synthesis. Representative examples of such promoters, include the LTR or SV40 promoter, the *E .coli* lac or trp, the phage lambda PL promoter and other promoters known to control expression of genes in prokaryotic or eukaryotic cells or in viruses. The promoter may also be the natural promoter of the outer membrane protein coding sequence. The expression vector also contains a ribosome binding site for translation initiation and a transcription terminator. Preferably, the recombinant expression vectors also include an origin of replication and a selectable marker, such as for example, the ampicillin resistance gene of *E. coli* to permit selection of transformed cells, i.e. cells that are expressing the heterologous DNA sequences.

The polynucleotide sequence encoding the outer membrane protein is incorporated into the vector in frame with translation initiation and termination sequences, and optionally, with a leader sequence capable of directing secretion of the translated protein into the periplasmic space or extracellular medium. Optionally, the sequence encodes a fusion outer membrane protein which includes an N-terminal or C-terminal peptide or tag that stabilizes or simplifies purification of the expressed recombinant product. Representative examples of such tags include

sequences which encode a series of histidine residues, the Herpes simplex glycoprotein D, or glutathione S-transferase.

Suitable hosts cells are, for example, bacterial cells, such as *E coli,* fungal cells, such as yeast; and animal cells such as Chinese Hamster Ovary cells. Host cells are transformed with the expression vectors of this invention and cultured in conventional nutrient media. Such media optionally contains additional compounds, such as for example compounds that induce promoters, such as for example isopropyl-β-D-thiogalactoside which induces the Lac promoter, or compounds, such as for example, ampicillin, which allows for selection of transformants.

In addition to serving as a template in the production of the outer membrane, the polynucleotide which encodes the outer membrane protein is useful as a probe for isolating other genes that encode the outer membrane proteins of other ehrlichiae species. The outer membrane protein encoding polynucleotide or smaller portions thereof, such as for example oligonucleotides of 200 to 2000 nucleotides can also be radiolabeled and used as hybridization probes.

The proteins OMP-1A, OMP-1B, OMP-1C, OMP-1D, OMP-1F from E. chafeenis and the OMP from E. Canis

The present invention also the proteins OMP-1A, OMP-1B, OMP-1C, OMP-1D, OMP-1F from E. chafeenis and the OMP from E. Canis. In one embodiment, OMP-1A, OMP-1B, OMP-1C, OMP-1D, OMP1-E, and OMP-1F have the amino acid sequences shown in FIG. In one embodiment the outer membrane protein from E. canis has the amino acid sequence shown in Fig. In other embodiments the respective outer membrane protein comprises an allelic variant or a derivative of the amino acid sequence s shown in Fig. 4, 8, and 14. In another embodiment the outer membrane protein is a fusion protein that further comprises additional amino acids fused to the amino terminus or carboxy terminus of PMR. The additional amino acids aid in, for example, purification of the protein. In addition to naturally occurring allelic forms, the outer membrane protein as described herein embraces non-naturally occurring allelelic forms of outer membrane , where one or more of the amino acids have been replaced by conservative amino acid residues, typically by using direct synthesis or recombinant techniques.

Preparing the Outer Membrane Proteins

The outer membrane proteins of the present invention are synthetically produced by conventional peptide synthesizers. The outer membrane proteins are also produced using cell-free translation systems and RNA molecules derived from DNA constructs that encode the outer membrane protein. Alternatively, the outer membrane protein is made by transfecting host cells with expression vectors that comprise a DNA sequence which encodes the outer membrane protein and then inducing expression of the outer membrane protein in the host cells.

The outer membrane protein is expressed in suitable host cells, such as for example, mammalian cells, yeast, bacteria, or other cells under the control of suitable promoters. Following transformation of the suitable host strain and growth of the host strain to an appropriate cell density, the cells are typically harvested by centrifugation, disrupted by physical or chemical means, and the resulting crude extract retained for further purification of the outer membrane protein.

The recombinant outer membrane protein that is expressed in a host cell culture is usually isolated by initial extraction from cell pellets or from cell culture medium, followed by salting-out, and one or more chromatography steps, including aqueous ion exchange chromatography, size exclusion chromatography steps, and high performance liquid chromatography (HPLC).

The outer membrane proteins, particularly OMP-1A, and the 30 kDa protein from E. canis are useful for preparing antibodies for immunolabeling isolates of E. chafeenis and E. canis respectively. Such antibodies are useful tools for diagnosing human ehrlichiosis and canine ehrlichiosis, respectively. The outer membrane proteins, particularly OMP-1A, and the 30 kDa protein from E. canis are useful for preparing vaccines to protect humans and dogs respectively from ehrlichiosis.

Antigens:
1.E.chaffeensis recombinant 28 kDa protein
2.Purified E.canis, Oklahoma strain
3.Purified E.canis, Cujo or others
4.E.canis recombinant 30 kDa protein

Antisera:
1. Dog anti-E.chaffeensis, Oklahoma strain serum
2. Normal dog serum
3. Human anti-E.chaffeensis serum
4.Normal human serum
5.Clinical E.canis IFA positive sera
6.Clinical E.canis IFA negative sera
7.Experimentally infected dog sera

Reagents:
1. PBS
2.T-PBS: 0.05% Tween 20 PBS
3.Secondary antibody:
Peroxidase conjugated anti-dog IgG
Peroxidase conjugated anti-dog IgM
Peroxidase conjugated anti-human IgG
Peroxidase conjugated anti-human IgM
4.Peroxidase color development solution
3,3'-diaminobenzidine.4 HCl (Nakaraikagaku-don't use others) 0.3g
CH3COONa. 3H2O 1.0 g
Dist H2O 100 ml
1N-NaOH approximately 2.5 ml to make pH 6.2 .
3 % H2O2 0.7 ml
Make one day before reaction. Cover the bottle with aluminum foil to prevent light. Keep in the dark. Before use filter with filter paper. You can use it up to 1 week.
5. 0.4M H2SO4: Dilute the concentrated H2SO4 50 times.

Dot Immunoassay:
1. Cut nitrocellulose membrane to fit in the apparatus. Never touch the membrane with your finger. The membrane is brittle and hand carefully.
2. Soak the membrane in PBS for 5 min by gently placing the sheet on the surface of PBS solution. Don't trap air bubbles.
3. Set the membrane in the dot-blot apparatus and aspirate for 5 min.
4. Carefully add antigen suspension (10 ul) in each well. Don't poke a hole in the membrane. Don't attach the droplet to the wall of the well
5. Cover the empty wells with salan wrap.
6. Aspirate for 5 min to make antigen bound.
7. Take out nitrocellulose membrane from the apparatus.
8. Soak the membrane in T-PBS at 37 C for 30 min.
9. Cut the membrane in strips. Cut the corner of the strip to identify the orientation.
10. Dry the membrane Place the membrane in the sandwich bag and keep it in a refrigerator.
11. Soak the dried membrane strip with antigen in T-PBS for 2-3 min.
12. Incubate the strip with the serum serially diluted. 3 ml /strip at room tem. for 60 min.
13. Wash the membrane in T-PBS for 3 times, 10 min each.
14. Incubate with peroxidase conjugated anti dog (or human) IgG or IgM diluted 2,000 fold in T-PBS at room tem. for 30 min.
15. Rinse the membrane like 13. Then rinse with dist H2O
16. Soak the membrane in developing solution for 1 min. or longer at room tem.
17. Soak the membrane in 0.4 M H2SO4 for 2 min to stop the reaction
18. Rinse with tap water.
19. Dry between filter paper.
20. Densitometric analysis of the membrane.

Fig 1

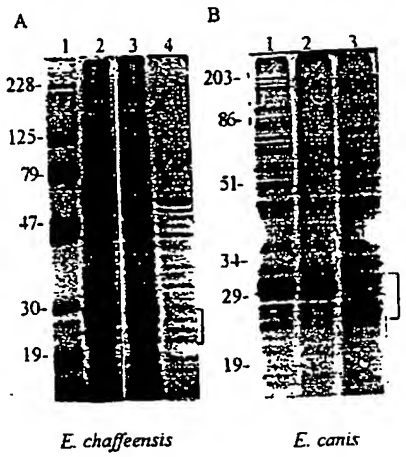


Fig. 2

Fig. 3 Fig. 3

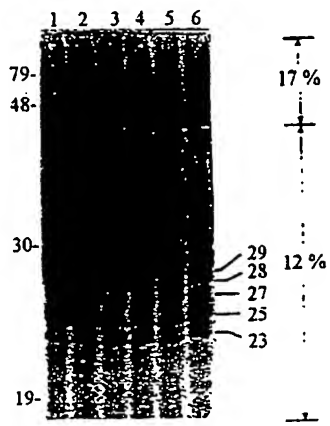


Fig 4A

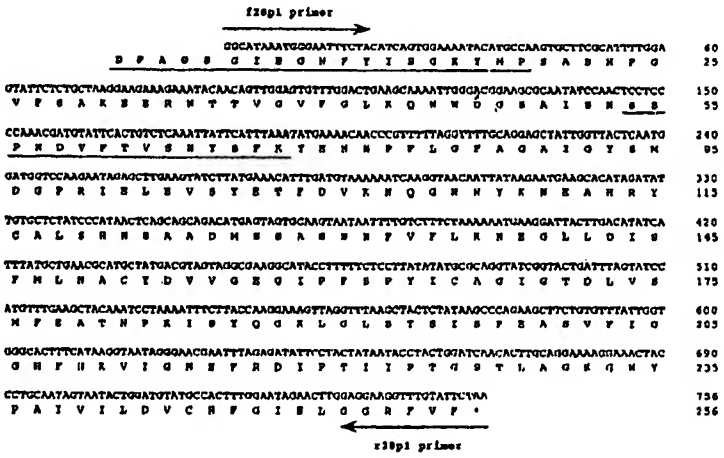
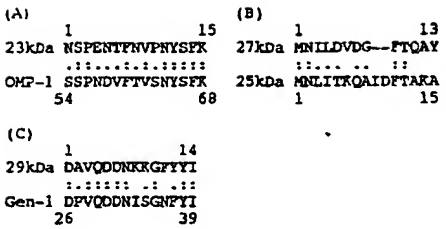


Fig 4B

Fig 5


(A)
1 15
23kDa NSPENTFNVPNYSFK
OMP-1 SSPNDVFTVSNYSFK
54 68
(B)
1 13
27kDa MNILDVDG--FTQAY
25kDa MNLITKQAIDFTARA
1 15
(C)
1 14
29kDa DAVQDDNKRGFYYI
Gen-1 DPVQDDNISGNFYI
26 39

60059353.091992

Fig 6

	10	20	30	40	50
OMP-1	DPAGSGIN--	-----GNFYI	SGKYMPSASH	FGVFSAKEER	NTTVGVFGLK
MAP-1	*VIQEEN*P-	----V*SV**	*A****T***	**KM*I**DS	RD*KA*****
MSP-4	S*MSHEVASE	GGVMG*S**V	GAA*S*APPS	VTS*DMR*SS	KE*SY*R*YD
	60	70	80	90	100
OMP-1	QNWDGSAISN	SSPNDVFTVS	NYSFKYENNP	FLGFAGAIGY	SMDGPRIELE
MAP-1	KD***VKTPS	GNT*SI**EK	D*********	*******V**	**N*****F*
MSP-4	KS---I*TID	V*VPAN*SK*	G*T*AFSK*L	ITS*D**V**	*LG*A*V***
	110	120	130	140	150
OMP-1	VSYETFDVKN	QGNNYKNEAH	RYCALSHNSA	ADMSSASNNF	VFLKNEGLLD
MAP-1	********R*	P*G****D**	H****DTA*S	STAGATTS--	*MV***N*T*
MSP-4	A**RR*ATLA	D*QYA*SG*E	SLA*ITR---	-*ANITET*Y	PVV*IDEITN
	160	170	180	190	200
OMP-1	ISFMLNACYD	VVGEGIPFSP	YICAGIGTDL	VSMFEATNPK	ISYQGKLGLS
MAP-1	**L*******	IMLD*M*V**	*V********	**VIN*****	L*******I*
MSP-4	T*V***G***	*LHTDL*V**	*V*****ASF	*DISKQVTT*	LA*R**V*I*
	210	220	230	240	250
OMP-1	YSISPEASVF	IGGHFHKVIG	NEFRDIPTI-	IPTGSTLAGK	GNYPAIV--I
MAP-1	***N****I*	******R***	***K**A*SK	VF*S*GN*SS	AVS*GFASA*
MSP-4	*QPT**I*LV	A**FY*GLFD	ESYK***AHN	SVKF*GE*KA	SVKAH*A---
	260				
OMP-1	LDVCHFGIEL	GGRFVF-			
MAP-1	*********I	******-			
MSP-4	----DY*FN*	*A**L*S			

Fig. 7

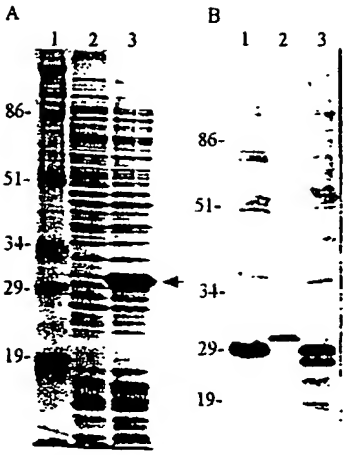
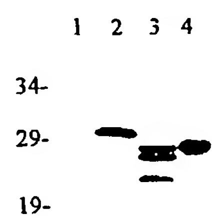
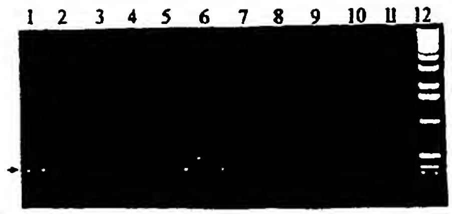


Fig. 8



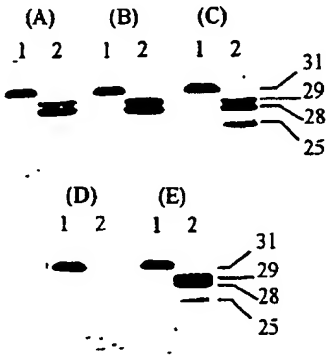
630

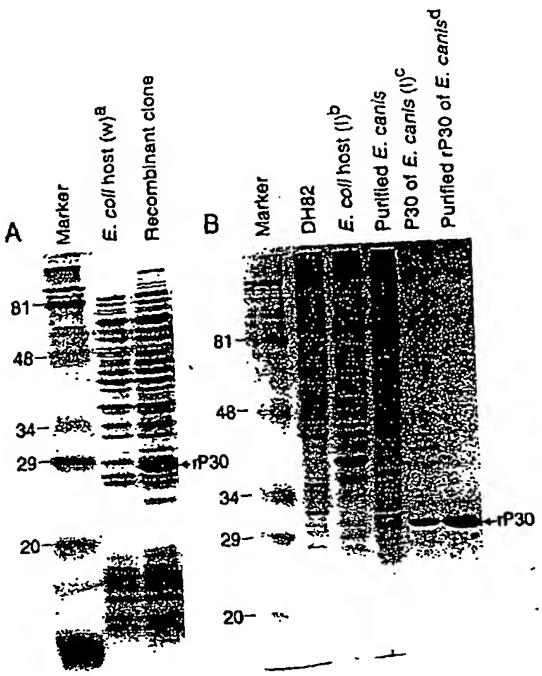
Fig. 9



630

Fig. 10





[a]Whole *E. coli* host lysate (w)
[b]Sonication-insoluble fraction (I) of *E. coli* host
[c]Sonication-insoluble fraction (I) of the recombinant clone (partially purified rP30)
[d]Highly purified rP30 using chromatography

Fig. 1 SDS-PAGE of *E. canis* recombinant rP30 (A) and the purified rP30 (B).

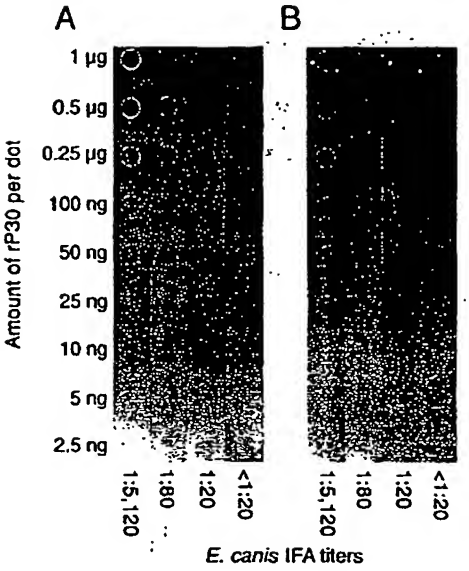
Fig 12

35

aSonication-insoluble fraction (I) of *E. coli* host
bSonication-insoluble fraction (I) of recombinant clone (partially purified rP30)
c*E. canis* IFA titers

Western blotting of purified *E. canis* and the recombinant P30 with clinical dog sera

Fig 13



Dot blot assay of *E. canis* rP30 using clinical dog sera of different IFA titers (A) and the dog sera preabsorbed with *E. coli* lysate (B)

GAATTC
Site ..... 579 , Select R1 1

```
   1 GGGATCAACGGGAACTTCTACATTAGTGCAAAGTATATGCCAAGTGCCTCACACTTTGGC    60
   1  G  I  N  G  N  F  Y  I  S  A  K  Y  M  P  S  A  S  H  F  G     20

  61 GTATTTTCAGTTAAAGAAGAGAAAAACACAACAACTGGAGTTTTCGGATTAAAACAAGAT   120
  21  V  F  S  V  K  E  E  K  N  T  T  T  G  V  F  G  L  K  Q  D     40

 121 TGGGACGGAGCAACAATAAAGGATGCAAGCAGCAGCCACACAATAGACCCAAGTACAATA   180
  41  W  D  G  A  T  I  K  D  A  S  S  S  H  T  I  D  P  S  T  I     60

 181 TTCTCCATTTCAAATTATTCATTTAAATATGAAAACAATCCATTTTTAGGGTTTGCAGGA   240
  61  F  S  I  S  N  Y  S  F  K  Y  E  N  N  P  F  L  G  F  A  G     80

 241 GCTATTGGCTACTCAATGGGTGGTCCAAGGGTAGAGTTTGAAGTGTCTTACGAAATATTT   300
  81  A  I  G  Y  S  M  G  G  P  R  V  E  F  E  V  S  Y  E  I  F    100

 301 GATGTAAAAAACCAAGGTAACAGTTACAAGAACGATGCTCACAAATATTGCGCTTTATCA   360
 101  D  V  K  N  Q  G  N  S  Y  K  N  D  A  H  K  Y  C  A  L  S    120

 361 AGACACACCGGAGGTATGCCACAAGCCGGTCATCAAAATAAATTTGTCTTCCTAAAAAAT   420
 121  R  H  T  G  G  M  P  Q  A  G  H  Q  N  K  F  V  F  L  K  N    140

 421 GAAGGATTACTTGACATATCACTTATGATAAACGCATGTTATGATATAACAATCGACAGC   480
 141  E  G  L  L  D  I  S  L  M  I  N  A  C  Y  D  I  T  I  D  S    160

 481 ATGCATTTTCTCCATATATATGTGCAGGTATTGGTAGTGACTTAGTTTCGATGTTTGAA   540
 161  M  P  F  S  P  Y  I  C  A  G  I  G  S  D  L  V  S  M  F  E    180

 541 ACTACAAATCCTAAAATTTCTTACCAAGGAAAGTTAGGTAAGCCGAATTC            579
 181  T  T  N  P  K  I  S  Y  Q  G  K  L  G                       193
```

SphI
R3
EcoRI
584/4

F1

P30

Fig 14a

GAATTC

Size 564 , Select 1

```
   1 TACATAAGTGGCAAATACATGCCAACAGCGTCACATTTTGGAATTTTTTCAGCTAAAGAA   60
   1  Y  I  S  G  K  Y  M  P  T  A  S  H  F  G  I  F  S  A  K  E   20

  61 GAACAAAGTTTTACTAAGGTATTAGTTGGGTTAGATCAACGATTATCACATAATATTATA  120
  21  E  Q  S  F  T  K  V  L  V  G  L  D  Q  R  L  S  H  N  I  I   40

 121 AACAATAATGATACAGCAAAGAGTCTTAAGGTTCAAAATTATTCATTTAAATACAAAAAT  180
  41  N  N  N  D  T  A  K  S  L  K  V  Q  N  Y  S  F  K  Y  K  N   60

 181 AACCCATTTCTAGGATTTGCAAGAGCTATTGGTTATTCAATAGGCAATTCAAGAATAGAA  240
  61  N  P  F  L  G  F  A  R  A  I  G  Y  S  I  G  N  S  R  I  E   80

 241 CTAGAAGTATCACATGAAATATTTGATACTAAAAACCCAGGAAACAATTATTTAAATGAC  300
  81  L  E  V  S  H  E  I  F  D  T  K  N  P  G  N  N  Y  L  N  D  100

 301 TCTCACAAATATTGCGCTTTATCTCATGGAAGTCACATATGCAGTGATGGAAATAGCGGA  360
 101  S  H  K  Y  C  A  L  S  H  G  S  H  I  C  S  D  G  N  S  G  120

 361 GATTGGTACACTGCAAAAACTGATAAGTTTGTACTTCTGAAAAATGAAGGTTTACTTGAC  420
 121  D  W  Y  T  A  K  T  D  K  F  V  L  L  K  N  E  G  L  L  D  140

 421 GTCTCATTTATGTTAAACGCATGTTATGACATAACAACTGAAAAAATGCCTTTTTCACCT  480
 141  V  S  F  M  L  N  A  C  Y  D  I  T  T  E  K  M  P  F  S  P  160

 481 TATATATGTGCAGGTATTGGTACTGATCTCATATCTATGTTTGAGACAACACAAAACAAA  540
 161  Y  I  C  A  G  I  G  T  D  L  I  S  M  F  E  T  T  Q  N  K  180

 541 ATATCTTATCAAGGAAAGTTAGGTAAG                                   564
 181  I  S  Y  Q  G  K  L  G                                       188
```

Nde I

EcoRI

F2

p30a.

Fig 14b.

INTRODUCTION

Ehrlichia chaffeensis, which causes human monocytic ehrlichiosis, is an obligatory intracellular bacterium of monocytes and macrophages, and belongs to the family *Rickettsiaceae*. Human ehrlichiosis was first reported in the United States in 1987 (21). Most patients have a history of tick exposure and develop a febrile illness similar to Rocky Mountain spotted fever. Since 1987, over 400 cases of human ehrlichiosis, detected primarily by serological means, have been reported in 30 states (3, 14, 16).

Recently, several protein antigens of *E. chaffeensis* were identified by western blot analysis using naturally-infected human, experimentally-inoculated dog sera, or monoclonal antibodies (7-10, 13, 31, 35, 40-42). Two of these antigens, namely, heat shock protein (HSP) 60 homolog (35) and 120-kilodalton (kDa) protein (41, 42), have been cloned, sequenced and expressed. Two proteins ranging from 28 to 30 kDa of *E. chaffeensis* were shown to be dominant antigens and were cross-reactive between two *Ehrlichia* spp.: *E. chaffeensis* and *E. canis* (7, 31). Studies with monoclonal antibodies against *E. chaffeensis* showed that 2 to 3 proteins from 22 to 30 kDa react with 3 monoclonal antibodies by westernblotting and that these antigens are exposed on the surface of the organism by innunogold labeling of negatively-staining ehrlichiae (8-10, 40). However, why multiple proteins of different molecular sizes react with the monoclonal antibodies has not been answered. These antigens in the 30-kDa range of *E. chaffeensis* were not examined at the molecular level.

In this study, we demonstrated that a potentially immunoprotective 28-kDa protein (designated P28) located on *E. chaffeensis* surface and along with antigenically cross-reactive proteins in 30-kDa range, are encoded by a multigene family.

MATERIALS AND METHODS

Organisms and purification. *E. chaffeensis* Arkansas strain and *E. canis* Oklahoma strain were cultivated in the DH82 dog macrophage cell line (31) and purified by Percoll density

gradient centrifugation as described elsewhere (33, 38).

Preparation of the ehrlichial outer membrane fraction. The procedure for *Orientia tsutsugamushi* was followed with modifications (26). Briefly, purified ehrlichiae (100 µg) were suspended with 10 mM sodium phosphate buffer, pH 7.4, containing 0.1% Sodium *N*-lauroyl sarcosine (Sarkosyl) [Sigma, St. Louis, MO], 50 µg/ml each DNase I (Sigma) and RNase A (Sigma), and 2.5 mM $MgCl_2$. After incubation at 37°C for 30 min, the sample was separated by centrifugation at 10,000 x g for 1 h into the soluble supernatant and the insoluble precipitate. The insoluble pellet was resuspended 2 to 3 times with 0.1% Sarkosyl and centrifuged. The final pellet was analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE), as described elsewhere (32), and by electron microscopy. The pellet was used as the ehrlichial outer membrane fraction. To investigate contamination by ehrlichial inner membrane, succinic dehydrogenase activity was examined as described elsewhere (11).

Analysis of the N-terminal amino acid sequence of outer membrane proteins in the 30-kDa range. Proteins in the Sarkosyl-insoluble pellet prepared from 400 µg of purified *E. chaffeensis* were separated by a reversed-discontinuous (Rd) SDS-PAGE (2.5-cm-long 17% gel on top of 11-cm-long 12% gel) and electrophoretically transferred to a ProBlot™ membrane (Applied Biosystems, Foster City, CA) as described elsewhere (44). The portion of the membrane containing bound proteins was excised and analyzed with an Applied Biosystems protein sequencer (Model 470).

Primer design for amplification of a *p28* gene encoding a 28-kDa major protein (P28) of *E. chaffeensis*. The N-terminal amino acid sequence of P28 (one of the major proteins separated by RdSDS-PAGE as described above) was determined as D P A G S G I N G N F Y S G K Y M P. We designed a forward primer, FECH1, based on 6th to 12th amino acids of this sequence: 5'-CGGGATCCGAATTCGG(A/T/G/C)AT(A/T/C)AA(T/C)GG

(A/T/G/C)AA(T/C)TT(T/C)TA -3'. Amino acids at the 1 to 5 positions of the N terminus of P28 were not included in this primer design to increase annealing efficiency, since Ser with 6 codons was present at the 5 position. For insertion into an expression vector, a 14-bp sequence (underlined) was added at the 5' end of primer to create an *Eco*RI and a *Bam*HI site.

A reverse primer was designed from two proteins which we found to be related to P28 based on N-terminal amino acid sequence comparison. One of the proteins was the *Cowdria ruminantium* major antigen protein (MAP)-1. The C-terminal sequence of the MAP-1 is (N terminus) . . . G G R F V F * (C terminus) [*: termination codon] (36). Another protein was the *Anaplasma marginale* major surface protein (MSP)-4 (24), of which the entire amino acid sequence is homologous to that of *C. ruminantium* MAP-1 (36). The C-terminal sequence of the MSP-4 is (N terminus) . . . G A R F L F S * (C terminus). An oligonucleotide primer, RECH2, complementary to a DNA sequence corresponding to amino acid sequence conserved between the C termini of the MAP-1 and the MSP-4, (N terminus) G (G/A) R F (V/L) F * (C terminus), was prepared with the addition of a 9-bp sequence (underlined) including a *Not*I site at the 5' end for ligation into an expression vector : 5'-AGCGGCCGCTTA(A/G)AA(T/C)A(C/G) (A/G)AA (C/T)CT T(C/G)C TCC -3'.

Cloning, sequencing, and expression of the *p28* gene. Genomic DNA of *E. chaffeensis* was isolated from purified organisms as described elsewhere (25). PCR amplification with FECH1 and RECH2 primers was performed using a Perkin-Elmer Cetus DNA Thermal Cycler (model 480). A 0.8-kb amplified product was cloned in the pCRII vector of a TA cloning kit, as described by the manufacturer (Invitrogen Co., San Diego, CA). The clone obtained was designated pCRII*p28*. Both strands of the inserted DNA were sequenced by a dideoxy-termination method with an Applied Biosystems 373A DNA sequencer.

The 0.8-kb *p28* gene was excised from the clone pCRII*p28* by *Eco*RI-*Not*I double-digestion, ligated into *Eco*RI-*Not*I sites of a pET 29a expression vector, and amplified in *Escherichia coli* BL21(DE3)pLysS (Novagen, Inc., Madison, WI). The clone (designated

pET29*p28*) produced a fusion protein with a 35-amino acid sequence carried from the vector at the N terminus.

Antisera and western blot analysis. Convalescent-phase serum from a patient with clinical signs of human ehrlichiosis was used as described previously (31). For preparation of the rabbit anti-recombinant (r) P28 antibody, the gel band corresponding to the rP28 in SDS-PAGE was excised without staining, minced in phosphate-buffered saline (PBS), pH 7.4, and mixed with an equal volume of Freund's incomplete adjuvant (Sigma). The mixture (1 mg of protein each time) was subcutaneously injected into a rabbit every 2 weeks four times. Antibody titers of the patient serum and the rabbit anti-rP28 antibody were determined to be 1:2,560 and 1:1,280 against *E. chaffeensis* antigen by indirect immunofluorescence assay (IFA) as described elsewhere (30).

Western blot analyses were performed with 1:1,000 dilutions of these sera by a procedure described elsewhere (32). Alkaline phosphatase-conjugated affinity-purified anti-human, anti-rabbit or anti-mouse immunoglobulin G (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD) were used at a 1:1,000 or 1:2,000 dilution as secondary antibodies.

Immunoelectron microscopy. *E. chaffeensis*-infected DH82 cells were sonicated and centrifuged at 400 x g for 10 min. The supernatant was then centrifuged at 10,000 x g for 10 min to obtain ehrlichia-enriched pellet. The pellet was resuspend and incubated with rabbit anti-rP28 antibody or normal rabbit serum (1:100 dilution) at 37°C for 1h in PBS containing 1 % bovine serum albumin (BSA-PBS). After washing, the ehrlichiae was incubated with gold-conjugated protein G (20 nm, Sigma) at 1:30 dilution for 1 h at room temperature in BSA-PBS. After washing again, the specimen was fixed with 1.25% formaldehyde, 2.5% glutaraldehyde, and 0.03% trinitrophenol in 0.1 M cacodylate buffer (pH 7.4) for 24h and postfixed in 1% osmium-1.5% potassium ferricyanide for 1 h (34). The section was then embedded in PolyBed 812 (Polysciences, Warrington, Pa). The specimen was ultrathin sectioned at 60 nm, stained with uranyl acetate and lead citrate, and observed with a Philips 300 transmission electron microscope at

60 kV.

Southern blot analysis. Genomic DNA extracted from the purified *E. chaffeensis* (200 ng each) was digested with restriction endonucleases, electrophoresed, and transferred to Hybond-N^+ nylon membrane (Amersham, Arlington Heights, IL), by a standard method (23). The 0.8-kb *p28* gene fragment from the clone pCRII*p28* was labeled with [α-^{32}P]dATP by the random primer method using a kit (Boehringer Mannheim, Indianapolis, IN) and the lebeled fragment was used as a DNA probe. Hybridization was performed at 60°C in rapid hybridization buffer (Amersham) for 20 h. The nylon sheet was washed in 0.1 x SSC (1 x SSC containing 0.15M sodium chloride and 0.015M sodium citrate) with 1% SDS at 55°C and the hybridized probes were exposed to Hyperfilm (Amersham) at - 80°C.

Cloning and sequencing of genomic copies of *E. chaffeensis p28* gene. The *EcoR* I and *Pst* I fragments of DNA, detected by genomic Southern blot analysis as described above, were inserted into pBluescript II KS (+) vectors, and the recombinant plasmids were introduced into *E. coli* DH5α. Using the colony hybridization method (23) with the ^{32}P-labeled *p28* gene probe, four positive clones were isolated from the transformant. The positive clones were designated pEC2.6, pEC3.6, pPS2.6, and pPS3.6. These contained the ehrlichial DNA fragments of 2.6-kb (*Eco*R I), 3.6 kb (*Eco*R I), 2.6 kb (*Pst* I), and 3.6 kb (*Pst* I), respectively. The inserts of the clones pEC3.6 and pPS2.6 overlapped as shown in Fig. 5. The overlapping area was further confirmed by PCR of *E. chaffeensis* genomic DNA with two pairs of primer sets interposing the junctions of the four clones (Fig. 7). The 1.1- to 1.6-kb DNA fragments of *Hin*dIII-*Hin*dIII, *Hin*dIII-*Eco*RI, or *Xho*I-*Eco*RI in the pEC2.6 and pEC3.6 were subcloned for sequencing. DNA sequencing was performed with suitable synthetic primers by dideoxy-termination method as described above.

Immunization of mice and *E. chaffeensis*-challenge. The rP28 band in SDS-

PAGE was excised, minced, and mixed with an equal volume of Freund's incomplete or complete adjuvant. Nine BALB/c male mice (6 weeks old) were divided into two groups. Five mice were intraperitoneally immunized a total of four times at 10-day intervals; twice with a mixture of the minced gel with the rP28 (30 to 40 μg of protein per mouse each time) and incomplete adjuvant, and twice with a mixture of the recombinant protein (the same amount as before) and complete adjuvant. Four mice were intraperitoneally injected with a mixture of the minced gel without protein and the respective adjuvants. For ehrlichia-challenge, approximately 1 x 10^7 DH82 cells heavily-infected with *E. chaffeensis* were disrupted by sonication in serum-free DMEM (GIBCO-BRL) and centrifuged at 200 x g for 5 min. The supernatant was diluted to a final volume of 5 ml, and 0.3 ml was inoculated intraperitoneally into each mouse 10 days after the last immunization.

Detection of *E. chaffeensis* 16S rDNA in *Ehrlichia*-challenged mice. At day 5 post-challenge, approximately 1 ml of blood was collected in an EDTA tube from each mouse. Total DNA was prepared from 0.2 ml of the buffy coat from the blood with a QIAamp blood kit (QIAGEN, Inc., Chatsworth, CA), and was used as the template for PCR detection of *E. chaffeensis* 16S rDNA. The PCR detection by primers HE1 (5'-CAATTGCTTATAACCTTTTGGT TATAAAT-3') and HE3 (5'-TATAGGTACCGTCATTATCTTCCCTAT - 3'), which yield a 389-bp fragment specific to *E. chaffeensis* 16S rDNA (4), was performed as described previously (39). The procedure allows detection from ≥10 pg of genomic DNA from purified *E. chaffeensis*.

Sequence analysis and GenBank accession number. Nucleotide sequences were analyzed with the DNASIS program (Hitachi Software Engineering Co., Ltd., Yokohama, Japan). A homology search was carried out with databases of the GenBank, Swiss Plot, PDB and PIR by using the software basic local alignment search tool (2) in the BLAST network service (the National Center for Biotechnology Information, Bethesda, MD). Phylogenetic analysis was

performed by using the PHYLIP software package (version 3.5) [17]. An evolutional distance matrix, generated by using the Kimura formula (17) in the PROTDIST, was used for construction of a phylogenetic tree by using the unweighted pair-group method analysis (UPGMA) [17]. The data were also examined using parsimony analysis (PROTPARS in PHYLIP). A bootstrap analysis was carried out to investigate the stability of randomly generated trees by using SEQBOOT and CONSENSE in the same package. The nucleotide sequence of the *p28* gene and its gene copies has been assigned GenBank accession numbers U72291 and AF021338, respectively.

RESULTS

Identification of major outer membrane proteins of *E. chaffeensis*. The ehrlichial outer membrane fraction was prepared from Percoll-purified *E. chaffeensis* by Sarkosyl treatment. Transmission electron microscopy revealed that the purified ehrlichial fraction consists of a mixture of electron dense and light forms of *E. chaffeensis* with slight disintegration of inner membrane (Fig. 1A). Ehrlihciae were not surrounded with the host inclusion membrane. Various sizes of membrane vesicles (< 1 μm) without significant ribosomes or nuclear materials were observed in the Sarkosyl-insoluble fraction from the organism (Fig. 1B). Succinic dehydrogenase (inner membrane marker enzyme of gram negative bacteria) activities were at less than the detection limit (1 n moles / min / mg of protein) in the Sarkosyl-insoluble fraction compared to approximately 10 n moles / min / mg of protein in the Percoll-purified organisms, suggesting that the insoluble fraction primarily consisted of the outer membrane of *E. chaffeensis*.

Analysis of the Sarkosyl-soluble and insoluble fraction of *E. chaffeensis* by SDS-PAGE suggested that proteins of 30-kDa range in the insoluble fraction represent the major outer membrane proteins of this organism (Fig. 2A). *E. canis* was antigenically cross reactive with *E. chaffeensis* (7, 31). A similar result was obtained in the case of *E. canis* using the same procedure with Sarkosyl (Fig. 2B). These findings indicate that the 30-kDa range proteins represent the major

outer membrane proteins of these two *Ehrlichia* spp. Since it was impossible to resolve overlapping protein bands in the 30-kDa range of *E. chaffeensis* by conventional SDS-PAGE, RdSDS-PAGE was performed and at least five proteins of 30-kDa range in *E. chaffeensis* (P23, P25, P27, P28, and P29 designated from the molecular sizes in Fig. 2C) could be resolved from the Sarkosyl-insoluble proteins.

Cloning, Sequencing and expression of a *p28* gene encoding *E. chaffeensis* P28. A 0.8-kb *p28* gene, amplified by PCR, was cloned and sequenced as described in Materials and Methods (Fig. 3). The 0.8-kb DNA fragment, cloned in pCRII*p28*, had an open reading frame (ORF) of 756 bp encoding a 251-amino acid protein (including both PCR primer regions) with a molecular mass of 27,685 Da (Fig. 3). *E. coli* transformed with pET29*p28* expressed a 31-kDa recombinant (r) P28 (Fig. 4A) which was larger than native P28 in size because of the fusion protein which a 35-amino acid sequence including the S.Tag peptide (20) derived from a pET expression vector was located at the N terminus. Serum from a patient with clinical signs of human ehrlichiosis reacted strongly to rP28 (31 kDa) in *E. coli*, P28 and P29 in *E. chaffeensis*, and also P30 in *E. canis* (Fig. 4B). The rabbit anti-rP28 antibody recognized not only rP28 (31 kDa) and P28, but also P29 and P25 of *E. chaffeensis* and P30 of *E. canis* (Fig. 4C), suggesting that P28 shares antigenic epitopes with P25 and P29 in *E. chaffeensis* and P30 of *E. canis*.

Immunoelectron microscopy. Transmission immunoelectron microscopy with colloidal gold-conjugated protein G and rabbit anti-rP28 antibody revealed gold particles bound to *E. chaffeensis* surface (Fig. 5). The distribution of the particles was random, close to the surface, and appeared as if almost embedded in the membrane, suggesting that the antigenic epitope protrudes very little from the lipid bilayer. Nonetheless, the antigenic epitope was surface-exposed, and thus, could be recognized by rabbit anti-rP28 antibody. No gold particles were observed on host cytoplasmic membrane or *E. chaffeensis* incubated with normal rabbit serum.

Identification and characterization of genomic copies of *E. chaffeensis p28*

gene. Genomic Southern blot analysis with several restriction enzymes resulted in one or more DNA fragment(s) of *E. chaffeensis* which could hybridize to ^{32}P-labeled *p28* gene probe (Fig. 6). The restriction enzymes used do not cut within the *p28* gene portion of the pCRII*p28* insert, and therefore, the Southern blot result shows that genes homologous to *p28* gene are present in the ehrlichial genome. *Xba* I, *Bgl* II, and *Kpn* I produced two bands, *Spe* I generated three bands, and *Eco*R V and *Pst* I produced multiple bands with different densities. *Eco*R I generated a broad band of 2.5 to 4 kb. These *p28* homologous genes are designated as *omp-1* (outer membrane protein-1) family.

Four DNA fragments from 2.6 to 3.6 kb were cloned from the *EcoR*I-digested and the *Pst*I-digested genomic DNA of *E. chaffeensis* by colony hybridization with radiolabeled *p28* gene probe. The inserted DNA of the two recombinant clones, pEC3.6 and pPS2.6, were overlapped as shown in Fig. 7. Sequencing revealed one 5'-truncated ORF of 243 bp (designated *omp-1*A) and five complete ORF of 836-861 bp (designated *omp-1*B to *omp-1*F), which are tandemly-arrayed and are homologous to the *p28* gene (but are not identical), in the ehrlichial genomic DNA of 6,292 bp (Fig. 8). The intergenic spaces were 581 bp between *omp-1A* and *omp-1B* and 260-308 bp among others. Putative promoter regions and ribosome-binding sites were identified in the noncoding regions (Fig. 8).

Structure of proteins encoded in the genes of the *E. chaffeensis omp-1* family. Five complete *omp-1* gene copies (*omp-1B* to *omp-1*F) encode 279 to 287-amino acid proteins with molecular masses of 30,320 - 31,508 Da. *Omp-1A* encodes an 82-amino acid partial protein (9,243 Da) which lacks the N-terminal region (Fig. 6). The 25-amino acid sequence at the N-terminus of OMP-1B to OMP-1F (encoded in *omp-1*B to *omp-1*F) is predicted to be a signal peptide because three carboxyl-terminal amino acids of the signal peptides (Ser-X-Ala in OMP-1B, Leu-X-Ser for OMP-C, and Ser-X-Ser for OMP-1D and OMP-1F) are included in the preferred amino acid sequence of signal peptidase at the processing sites proposed by Oliver (27). The

putative cleavage site of the signal peptide is shown by vertical arrowhead in Fig. 8. The calculated molecular masses of the mature OMP-1B to OMP-1F from the predicted amino acid sequences are 28,181 Da for OMP-1B, 27, 581 Da for OMP-1C, 28, 747 Da for OMP-1D, 27,776 Da for OMP-1E, and 27, 933 Da for OMP-1F. The estimated isoelectric points are 4.76-5.76 in the mature OMP-1B to OMP-1F. In Fig. 8, an underlined amino acid sequence in *omp-1F* gene (the 80th to 94th amino acids) was identical to the N-terminal amino acid sequences of *E. chaffeensis* native P23 protein as determined chemically, which indicates that P23 is derived from the *omp-1F* gene. Amino acid sequences identical to the N-terminal sequences of P25, P27, and P29 were not found in those from *omp-1* gene copies cloned in this study (data not shown).

Alignment of predicted amino acid sequences of the *E. chaffeensis* OMP-1s, including *Cowdria ruminantium* MAP-1 (36) which is related to the OMP-1 family, revealed substitutions or deletions of one or several contiguous amino acid residues throughout the molecules. The significant differences in sequences among the aligned proteins are seen in the regions indicated SV (semivariable region) and HV (hypervariable region) 1 to 3 in Fig. 9. Computer analysis for hydropathy revealed that protein molecules predicted from all *omp-1* gene copies contain alternative hydrophilic and hydrophobic motifs which are characteristic of transmembrane proteins. The HV1 and HV2 were found to locate in the hydrophilic regions (data not shown).

Similarities among amino acid sequences of the *E. chaffeensis* OMP-1s. The amino acid sequences of 5 mature proteins without signal peptides (OMP-1C to OMP-1F and a P28) were similar to one another (71-83%) but the sequence of OMP-1B was dissimilar to those of the 5 proteins (45-48%). The amino acid sequences of the 5 proteins showed an intermediate degree of similarity with that of *C. ruminantium* MAP-1 (59-63%), but the similarity between that of the OMP-1B and the *C. ruminantium* MAP-1 was low (45%). These relations are shown in a phylogenetic tree which was obtained based on the amino acid sequence alignment by UPGMA method in the PHYLIP software package (Fig. 10). Three proteins (P28, OMP-1D, and OMP-1F) and two proteins (OMP-1C and OMP-1E) formed two separate clusters. The OMP-1B was located

distantly from these two clusters. The *C. ruminantium* MAP-1 was positioned between the OMP-1B and other members in the OMP-1 family.

Protection against *E. chaffeensis*-challenge in rP28-immunized mice. To investigate whether immunization of rP28 induces protection against *E. chaffeensis* infection, 5 mice were immunized with rP28 and 4 mice were inoculated with acrylamide gel without the recombinant protein (control). Before challenge, all 5-immunized mice had a titer of 1:160 against *E. chaffeensis* antigen by IFA and all 4-nonimmunized mice were negative. Protection was assessed by PCR detection of *E. chaffeensis* 16S rDNA in the buffy coat of blood collected from the mice at 5 days postchallenge. *E. chaffeensis* can transiently establish infection in BALB/c mice. The infection is spontaneously cleared as *E. chaffeensis* cannot be reisolated in cell culture at day 10 postinfection (29). Day 5 is the optimum time at which establishment of ehrlichial infection can be examined by PCR without the influence of residual DNA from the ehrlichiae used as the challenge before the spontaneous clearance of organisms takes place. The *E. chaffeensis*-specific DNA fragment was observed in all nonimmunized mice but not in any immunized mice, indicating that immunization of rP28 apparently protects mice from ehrlichial infection (Fig. 11) and suggesting that the P28 is a potential protective antigen.

DISCUSSION

The outer membrane is the site where the host and ehrlichial interaction takes place. So far, the outer membrane fraction has not been prepared from any *Ehrlichia* spp., consequently the protein composition of outer membrane has been unknown. Using a Sarkosyl method, we identified five major proteins (P23 to P29) in the insoluble fraction of *E. chaffeensis*. Three of the five (P25, P28, and P29) were found to be antigenically cross reactive using anti-rP28 antibody and the antigenic epitopes were surface-located in *E. chaffeensis* as demonstrated by transmission immunoelectron microscopy. These observations, in addition to results of analysis by transmission electron microscopy and examination of succinic dehydrogenase activity in the Sarkosyl-insoluble

fraction, support that the procedure using Sarkosyl is useful for preparation of a fraction enriched in outer membrane of *E. chaffeensis*. Just like *Orientia tsutsugamushi* (26), the concentration of Sarkosyl required for *E. chaffeensis* was lower than those required for other facultative intracellular bacteria (6, 18, 37).

This is the first report that the major outer membrane proteins of *E. chaffeensis* in 30-kDa range are identified and characterized at molecular genetic level and protein sequence level. We and other investigators previously reported protein antigens of *E. chaffeensis* ranging from 22 to 30 kDa in sizes (7-10, 13, 31, 40). The two dominant antigens, P28 and P29 in the current study, seem to correspond to two proteins of 28 kDa and 30 kDa by Rikihisa et al. (31), and 28 kDa and 29 kDa by Chen et al. (7), respectively. In both previous studies, the antigens were recognized predominantly by convalescent-phase sera from human ehrlichiosis patients. The P28 and P29 may also correspond to proteins of 29 kDa and 30 kDa by Chen et al. (8), respectively, both of which were recognized by 7C1-B and 3C7 monoclonal antibodies (MAbs). The current study using the anti-rP28 antibody and the report by Chen et al. [8] using the MAbs indicated that the P28 (the current study) and the 29 kDa protein (Chen et. al. [8]) share antigenic epitopes with the P29 (the current study) and the 30 kDa protein (Chen et.al [8]), respectively. In the current study, the P25, P28, and P29 were recognized by anti-rP28 antibody.

E. canis 30-kDa protein was recognized by the antibody to rP28 of *E. chaffeensis* (the current study) and by the 7C1-B MAb to *E. chaffeensis* (Chen et al. [8, 10]). The 32-kDa MAP-1 of *C. ruminantium* (36) showed similarity with all members of the *E. chaffeensis* OMP-1 family in the amino acid sequences. The *C. ruminantium* MAP-1 also was cross reactive to a 27-kDa protein of *E. canis* (22),

By 16S rDNA sequence comparison, *E. chaffeensis*, *E. canis*, and *C. ruminantium* are closely related (12). Consequently, 30-kDa range proteins in the OMP-1 family may be common antigens among the three species in the tribe *Ehrlichieae*.

Using PCR-amplified *p28* gene as a probe, six similar genes were identified in *E. chaffeensis* genome. Genomic Southern blot result suggests the presence of additional *omp-1* gene copies. However, the precise number of the copies cannot be determined, since restriction site polymorphism in the gene copies may result in the production of several bands from a single copy.

Recently, in *Anaplasma marginale* which is related to *E. chaffeensis* by 16S rDNA sequence (12), two multigene families have been found (1, 28). In one of the families, multiple *msp-2* genes encoding a 36-kDa major surface protein constituted a minimum of 1% of the genome and were distributed widely throughout the chromosome. In addition, strain variations of the *msp-2* copies were demonstrated (28). In another family, *msp3* gene copies encoding a 63-kDa major surface protein also were distributed widely throughout the chromosome. The three unique *msp3* genes, *msp3-12*, *msp3-11*, and *msp3-19*, possessed a DNA sequence area homologous to that of *msp-2* within ORFs in the *msp3-12* and outside ORF in the *msp3-11* and *msp3-19* (1). It was found that the *omp-1* gene family of *E. chaffeensis* is different from those of *A. marginale*. First, the ORFs of *omp-1* gene copies are tandemly-arrayed in the genome. Second, amino acid sequences among the *omp-1* copies may have greater variation among those of *msp-2* copies of *Anaplasma*. The similarities were 45% to 83% among six *omp-1* copies whereas 95% between two *msp-2* copies identified (15). Strain variability similar to *A. marginale* may exist in *E. chaffeensis*, since strain variability of protein antigens with 7C1-B MAb has been reported by Chen et al. (8, 10).

In phylogenetic classification, three proteins (P28, OMP-1D, and OMP-1F) belong to the same cluster. The P23 (derived from *omp-1F* gene) which was identified in the *E. chaffeensis* outer membrane fraction also belongs to this cluster. It is unknown whether *omp-1D* and other gene copies in different clusters are silent genes. These genes at least are not actively expressed in *E. chaffeensis* cultured in *vitro*, since the products from the *omp-1* gene family except for P23, P25, P28, and P29, were not recognized in the Sarkosyl-insoluble outer membrane fraction.

We demonstrated that the rP28 protected mice from *E. chaffeensis* infection or accelarated

the spontaneous clearance of *E. chaffeensis*, suggesting that this or other *omp-1*-related proteins may be a protective antigen. Further molecular genetic studies are required for elucidating the mechanisms of the antigenic polymorphism or possible antigenic variation, i.e., whether selective expression of the *omp-1* gene copies are regulated at transcriptional level or by recombination events (gene conversions) among the unique gene repertoire, such as in the cases of the pili of *Neisseria gonorrhoeae* (19), *vmp* of *Borrelia hermsii* (5), and *vls* of *Borrelia burgdorferi* (43).

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FIGURE LEGENDS

FIG. 1. Transmission electron microscopy of Percoll-purified *E. chaffeensis* (A) and the insoluble precipitate after 0.1% Sarkosyl treatment of the organism (B). Note outer membrane vesicles of various sizes in B. (Bar = 1 μm).

FIG. 2. SDS-PAGE patterns of the insoluble precipitate and the soluble supernatant fraction after 0.1% Sarkosyl treatment of purified *E. chaffeensis* (A) and *E. canis* (B), and RdSDS-PAGE of major proteins in the 30-kDa range resolved from the Sarkosyl-insoluble pellet of *E. chaffeensis* (C). (A) Lanes: 1, Sarkosyl-soluble supernatant; 2, Sarkosyl-insoluble precipitate enriched with outer membrane; and 3, purified *E chaffeensis*. (B) Lanes: 1, Sarkosyl-soluble supernatant; 2, Sarkosyl-insoluble precipitate; and 3, purified *E. canis*. Both gels were stained with Coomassie blue. Brackets indicate a 30-kDa cluster of major outer membrane proteins. (C) The separation gel used consisted of a 17% gel on top of a 12% gel. The Sarkosyl-insoluble precipitate prepared from purified *E. chaffeensis* was blotted on a ProBlot™ membrane and stained with amido black (lanes 1-6). The protein bands present in each lane of the membrane were excised, and the N-terminal amino acid sequence of each protein was analyzed. Numbers on right or left of panels indicate molecular mass in kDa.

FIG. 3. DNA sequence and translation of *E. chaffeensis p28* gene cloned in pCRII*p28*. The N-terminal amino acid sequence of native P28 determined chemically is underlined. Five amino acid residues at the N terminus of P28 which were not included in the *p28* gene, are indicated by boldface. Arrows indicate annealing positions of the primer pair designed for PCR amplification of *p28* gene.

FIG. 4. Overexpression of *E. chaffeensis p28* gene (A), and western blot analysis with convalescent-phase serum from a human ehrlichiosis patient (B) and with a rabbit anti-recombinant (r) P28 antibody (C). (A) Lanes; M, molecular size markers; C, pET29a-transformed *E. coli* (negative control); R, pET29*p28*-transformed *E. coli*

(recombinant), and the recombinant (rP28) protein indicated by the arrowhead. (B and C) Lanes; Eca, purified *E. canis* ; R, pET29*p28*-transformed *E. coli*; and Ech, purified *E. chaffeensis*. A rabbit anti-rP28 antibody preabsorbed twice with pET29a-transformed *E. coli* at 37°C for 1 h each was used at a 1:300 dilution. Dominant protein antigens are schematically shown for designated molecular masses, P25 to P30, except for rP28 (31-kDa). Numbers indicate molecular mass in kDa.

FIG. 5. Transmission electron microscopy of *E. chaffeensis* immunogold-labeled with a rabbit anti-rP28 antibody. Protein G-gold particles (20 nm) are localized on the surface of the organism. (Bar = 0.1 μm).

FIG. 6. Genomic Southern blot analysis of *E. chaffeensis* with a ^{32}P-labeled 0.8-kb *p28* gene probe of the PCR□*p28* insert. Numbers indicate molecular size in kb.

FIG. 7. Restriction map of 6.3-kb genomic DNA including the *omp-1* gene copies in *E. chaffeensis* . The four DNA fragments were cloned from the genomic DNA (pPS2.6, pPS3.6, pEC2.6, and pEC3.6). A recombinant plasmid pPS2.6 has an overlapping sequence with that of pEC3.6. The closed boxes at the bottom show PCR-amplified fragments from the genomic DNA for confirmation of the overlapping area. Open boxes at the top indicate open reading frames (ORF) of *omp-1* gene copies with direction by arrows. Open boxes at the bottom show DNA fragments subcloned for DNA sequencing.

FIG. 8. DNA sequence of 6.3-kb genomic DNA of *E. chaffeensis* and the predicted amino acid sequences of *omp-1* gene copies. The sequence of putative ribosome-binding sites (RBS) and -10 and -35 promoter regions are underlined. The N-terminal amino acid sequence of *E. chaffeensis* P23 protein is underlined in the amino acid sequence of *omp-1F*. Vertical arrows show the putative cleavage site of the presumed signal peptide.

FIG. 9. Amino acid sequences alignment of seven *E. chaffeensis* OMP-1s and *Cowdria ruminantium* MAP-1. Aligned positions of identical amino acids with OMP-1F are

shown with dots. The sequence of *C. ruminantium* MAP-1 is from the report of Van Vliet et al. (36). Gaps indicated by dashes were introduced for optimal alignment of all proteins. Bars indicates semivariable region (SV) and three hypervariable regions (HV1, HV2, and HV3).

FIG. 10. Phylogenetic relationship among six members of the *E. chaffeensis* OMP-1 family and *Cowdria ruminantium* MAP-1. The evolutionary distance values were determined by the method of Kimura, and the tree was constructed by the UPGMA analysis. Scale bar = 5% divergence (in the amino acid sequences). The numbers at nodes are the proportions of 100 bootstrap resamplings that support the topology shown.

FIG.11. PCR detection of *E. chaffeensis* 16S rDNA fragment from *Ehrlichia*-challenged mice which were previously immunized with rP28 or nonimmunized. Template DNAs were prepared from blood buffy coats (0.2 ml) of all challenged mice. Arrow shows the *E. chaffeensis*-specific 16S rDNA fragment (389 bp) obtained by PCR amplification. Lanes: 1, positive control (using a total DNA from DH82 cells infected with *E. chaffeensis* as template); 2, negative control (PCR reaction without template); 3-6, nonimmunized mice; 7-11, immunized mice; and 12, DNA 1-kb ladder marker (GIBCO).

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Fig. 1

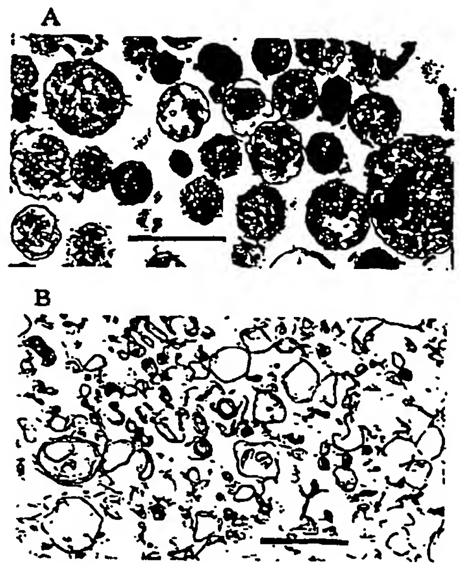

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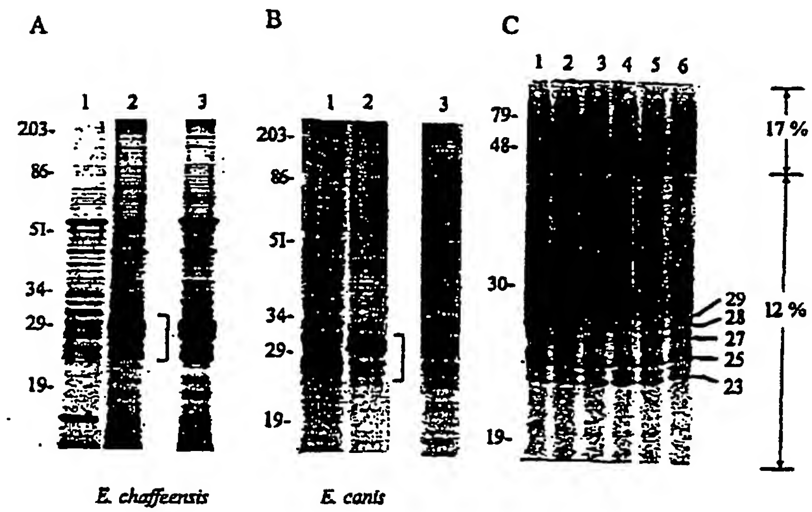
Fig. 2



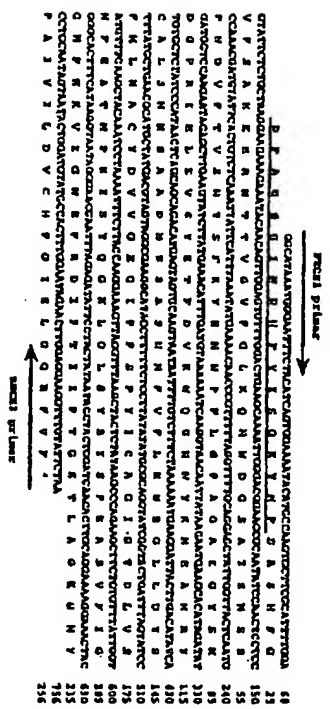


Fig 3

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Fig. 4

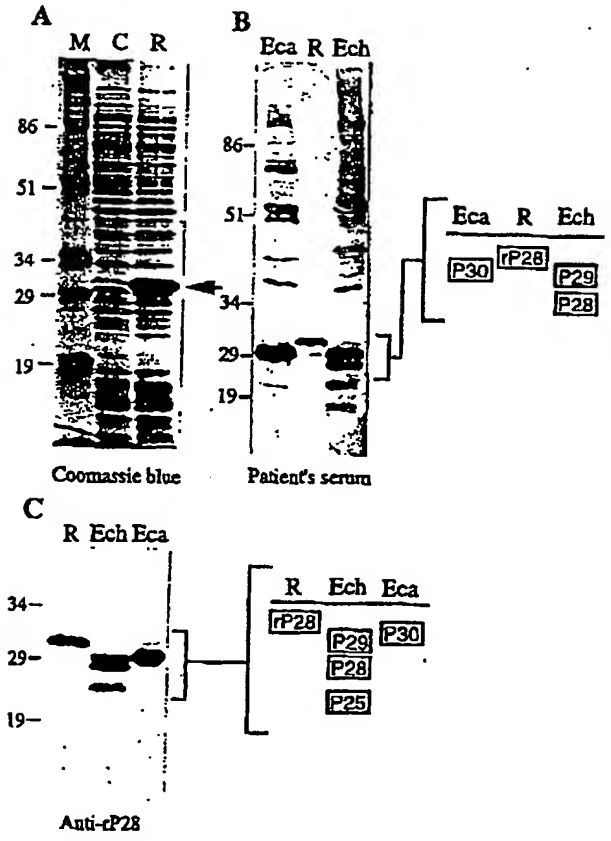
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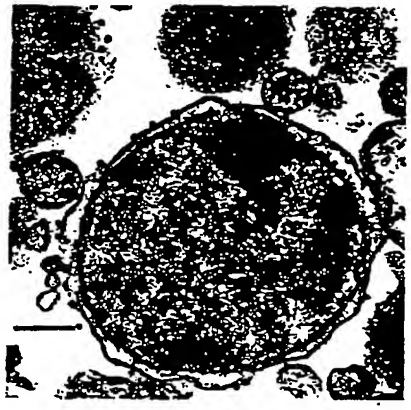
Fig. 5



Origene

Fig. 6

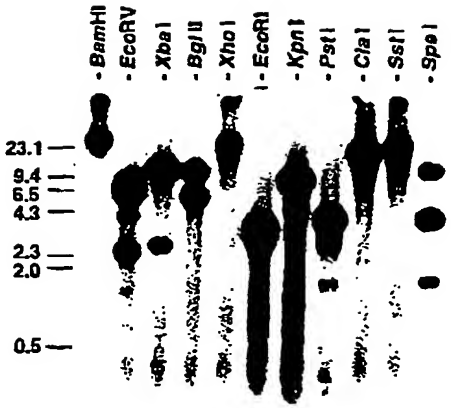


Fig. 2

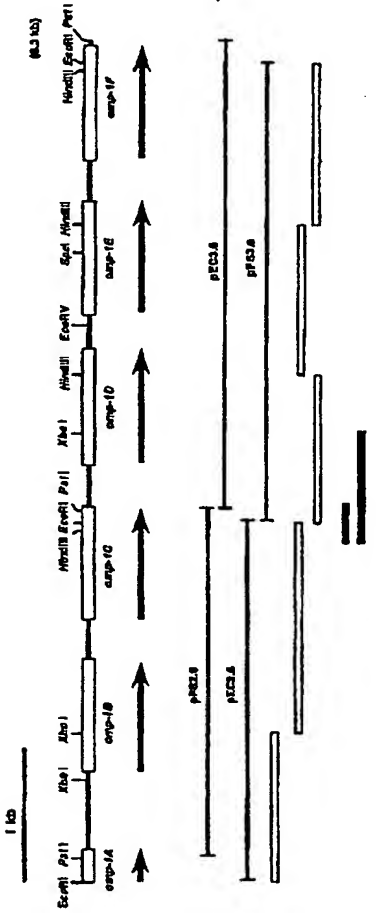


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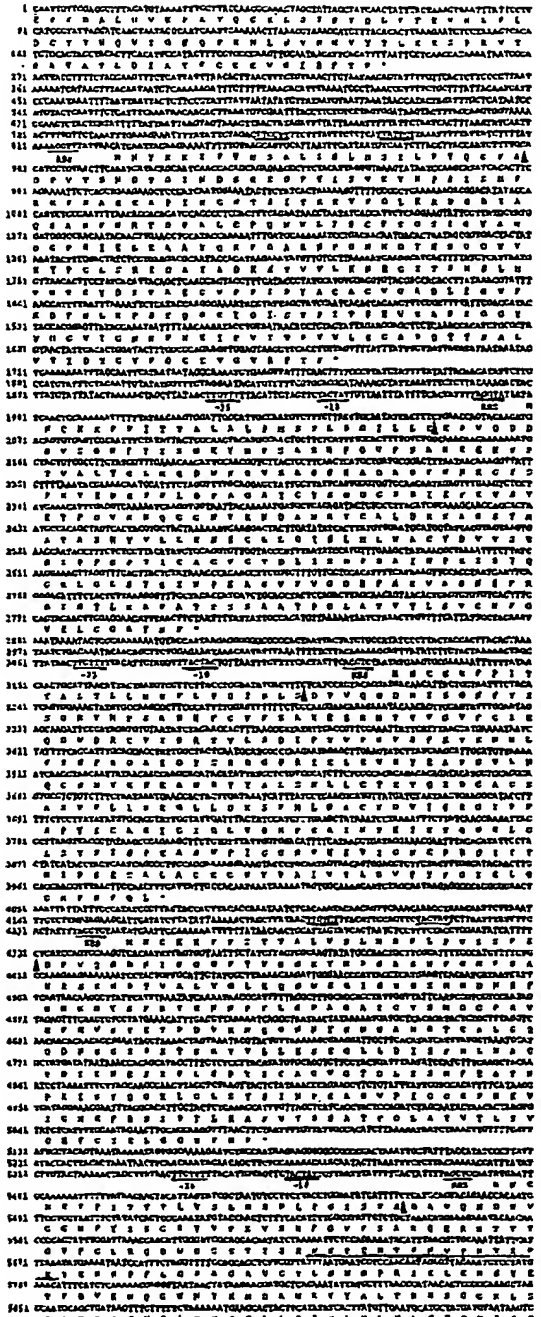
Fig. 8



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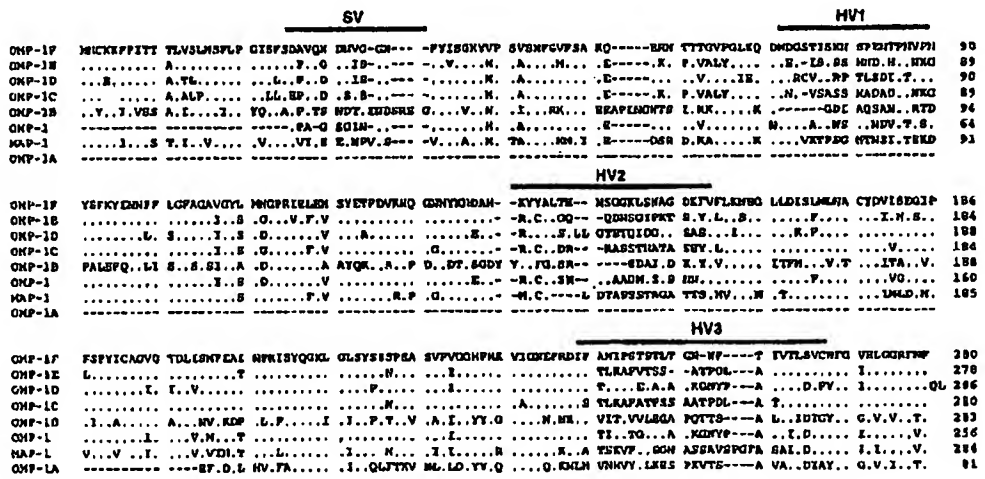

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Fig. 10

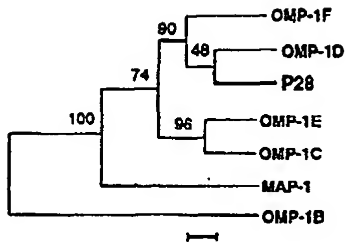

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Fig. 11.

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